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IDENTIFICATION AND ANALYSIS OF THE EXPRESSION OF THE AFLATOXIN BIOSYNTHETIC GENES NOR-1 AND VER-1 IN THE COMMERCIAL SPECIES ASPERGILLUS SOJAE AND A. ORYZAE AS WELL AS TOXIGENIC AND NON-TOXIGENIC STRAINS OF A. FLAVUS

presented by

Matthew David Rarick

has been accepted towards fulfillment
of the requirements for

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Dr. John E. Linz
Major professor

Date 5-2-96

IDENTIFICATION AND ANALYSIS OF THE EXPRESSION OF THE AFLATOXIN BIOSYNTHETIC GENES *NOR*-1 and *VER*-1 IN THE COMMERCIAL SPECIES *ASPERGILLUS SOJAE* AND *A. ORYZAE* AS WELL AS TOXIGENIC AND NON-TOXIGENIC STRAINS OF *A. FLAVUS*

By

Matthew David Rarick

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Department of Food Science and Human Nutrition

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ABSTRACT

IDENTIFICATION AND ANALYSIS OF THE EXPRESSION OF THE AFLATOXIN BIOSYNTHETIC GENES *NOR*-1 and *VER*-1 IN THE COMMERCIAL SPECIES *ASPERGILLUS SOJAE* AND *A. ORYZAE* AS WELL AS TOXIGENIC AND NON-TOXIGENIC STRAINS OF *A. FLAVUS*

By

Matthew David Rarick

Aflatoxins are a group of highly potent, carcinogenic, secondary metabolites produced by the imperfect fungi *Aspergillus flavus* and *A. parasiticus*. Previous studies have revealed that many species of *Aspergillus* have at least some of the genes involved in aflatoxin production and, in some cases, are able to use them to produce intermediates of the aflatoxin biosynthetic pathway. This study examined *A. sojae* (AS) and *A. oryzae* (AO) (commercial species used in enzyme production) as well as four *A. flavus* strains (aflatoxin producers 1059, 1273; non-producers 2112, 2115) for the presence of the aflatoxin pathway genes *nor*-1 and *ver*-1, for aflatoxin production, and for accumulation of *nor*-1 and *ver*-1 transcripts. Southern hybridization with the DNA probes *nor*-1 and *ver*-1 confirmed the presence of similar or identical genes in each strain. The fungal strains were then grown in duplicate to 48 and 72 hours as stationary and shake cultures in an aflatoxin inducing growth medium. The media from one set of duplicate cultures was analyzed for aflatoxin accumulation by means of enzyme linked immunosorbent assay (ELISA). Aflatoxin production by the *A. flavus* producers was detected, though at reduced levels compared to *A. parasiticus* SU-1, whereas, no toxin was ever detected from AS, AO, and the *A flavus* non-producers. The levels of aflatoxin produced in the stationary cultures

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ranged from 25 to 30 times more (per mg mycelia) than in the shake cultures. The mycelia were collected from the remaining set of duplicate cultures and the RNA extracted. Northern hybridization analysis with *nor*-1 and *ver*-1 probes showed that transcripts of the predicted size were present in the *A. flavus* producers at both time points in stationary cultures but lower levels were detected in the shake cultures. AS, AO, and the *A. flavus* non-producers did not produce detectable levels of transcript of the predicted size at either time point suggesting that *nor*-1 and *ver*-1 are not expressed. Identifying how non-toxigenic species differ from toxigenic species in the regulation of aflatoxin genes may provide some clues about the origin of the aflatoxin biosynthetic pathway, and the causes of the loss of toxin production in non producers. Ultimately, regulatory factors which effect aflatoxin synthesis may be identified and their production or activity manipulated to eliminate aflatoxin production.

To Fred.

cont

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ACKNOWLEDGMENTS

I would like to take this opportunity to thank all of those people who have made contributions not only to the work that I have done in the lab but also to those who have made a difference in any aspect of my life leading up to this publication.

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Literature Review

Overview

The aflatoxins are a group of secondary metabolites produced by the imperfect fungi *Aspergillus flavus*, *A. parasiticus*, and *A. nomius*. During the three decades since their initial discovery, 17 different aflatoxins have been identified (McLean and Dutton, 1995). Some of these chemicals have been found to be extremely carcinogenic, mutagenic, and teratogenic. Of these, aflatoxin B_1 (AFB_1) is the most potent (Ellis et al, 1991) and, in fact, is the most potent naturally occurring carcinogen known (based on animal studies) (Diener et al, 1987; CAST, 1979). Of the remaining 16 aflatoxins, aflatoxin G_1 (AFG_1) is the most toxic though it is far less reactive than AFB_1. The occurrence of aflatoxins in nature is dependant on where the fungal strains can grow. In general, AFB_1 is more common than the other aflatoxins and again AFG_1 comes in a distant second (Ellis et at, 1991).

The aflatoxins pose a health threat to all animals tested thus far. Adverse health effects have been seen in rats, monkeys, turkeys, and trout (to name a few test species) that have been fed a diet containing aflatoxins. This evidence has led to the hypothesis that aflatoxins may be dangerous to humans as well. This threat is not a primary concern in developed countries where foods found to be contaminated by aflatoxins can be discarded, rather it is an economic concern due to wasted product. In non-developed

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countries food may be scarce, therefore, even if contaminated, the food is eaten causing a potential health problem. Due to these two problems the elimination of aflatoxins from the food chain is a desired goal.

Several methods have been considered to achieve this goal. Among them, standard farming practices prior to harvest, and post harvest decontamination (to name a few) have been investigated and deemed unfeasible for reasons to be discussed later. One potential method being explored to eliminate aflatoxins is increasing host plant defenses by manipulating them genetically.

The Genus *Aspergillus*

P.A. Micheli first described species of *Aspergillus* in 1729 (Bossche et al, 1987; Micheli, 1729). He noted that the species of *Aspergillus* can be morphologically characterized by aerial hyphae rising out of septate mycelial cells. At the tip of each hypha there is a vesicle to which numerous sterigma are attached. Chains of conidia (spores) form from these sterigma (Figure 1a)(Bossche, 1987). The spores are cells which are dispersed via the air to allow propagation of each species. Micheli derived the name *Aspergillus* from the name aspergillum, a holy water sprinkler, due to the striking similarity in appearance (Figure 1b, c). Since Micheli's description the genus has grown. Work by Raper and Fennel identified 132 species in 1965 (Smith, 1994; Raper and Fennel, 1965). In 1984 Christensen and Tuthill proposed a total of 276 species and varieties in the genus *Aspergillus* (Smith, 1994; Cristensen and Tuthill, 1984). These species and varieties have been placed into 18 groups (for fast characterization) based on morphological and cultural characteristics.

Figure 1. Diagrams of *Aspergillus*. A) Diagram of conidiophore structure typically found in *Aspergillus* spp. B) First drawings of observed *Aspergillus* by Michelli. C) An aspergillum - a holy water sprinkler (Bossche, 1987).

Figure 1.

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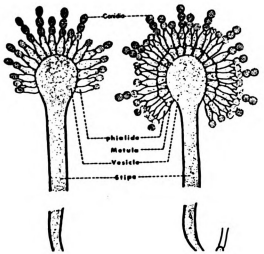
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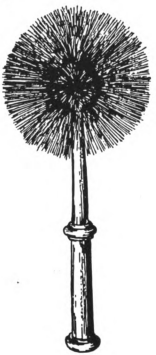

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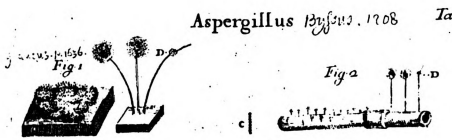
Aspergillus Micheli. 1708
Tab. 91
Fig. 1
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Fig. 2
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Many species within *Aspergillus* are of great importance to humans. Some species are beneficial. For example, some strains of *A. sojae* and *A. oryzae* have been used in the fermentation of shoyu (soy), miso, and sake for hundreds of years (Bossche, 1987; Smith, 1994). Also used in the food industry are enzymes such as α-amylase, glucoamylase, and lipase which are produced by strains of *A. niger*, *A. oryzae*, and *A. malleus* respectively (Smith, 1994) (Table 1).

Table 1. Some industrial *Aspergillus* species and the enzymes they produce (adapted from Smith, 1994)

A. awamori	Glucose oxidase
A. japonicus	Glycerol oxidase
A. niger	Amyloglucosidase
A. niger	β-Glucosidase
A. niger	β-Galactosidase
A. niger	Catalase
A. niger	Lipase
A. niger	Metalloproteinase
A. niger	Pectinase
A. oryzae	α-Amylase
Aspergillus species	β-Glucanase
Aspergillus species	β-Glucose dehydrogenase

By contrast, other species of *Aspergillus* are known to be harmful to humans. One way is the ability of some *Aspergillus* strains to cause pathogenic diseases in humans. The most common example of pathogenic *Aspergillus* is *A. fumigatus* (Bossche, 1987). Other

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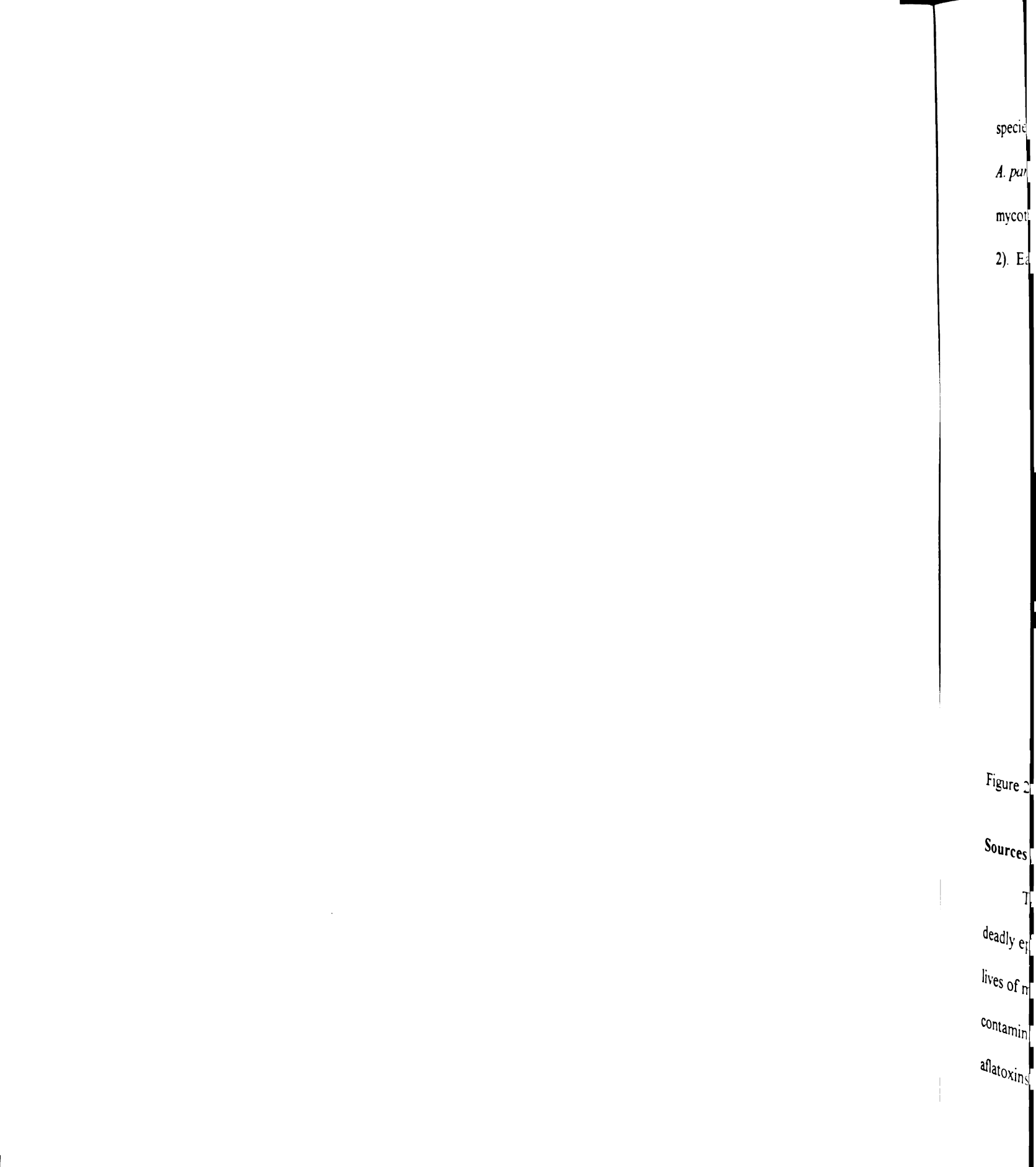

Figure 2

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species are harmful in that they produce toxic compounds. Species such as *A. ochraceus*, *A. parasiticus* and *A. flavus*, and *A. versicolor* present problems in that they produce the mycotoxins ochratoxin A, aflatoxins B_1 and G_1, and sterigmatocystin respectively (Figure 2). Each of these toxins has been hypothesized to be toxic to humans (Bossche, 1987).

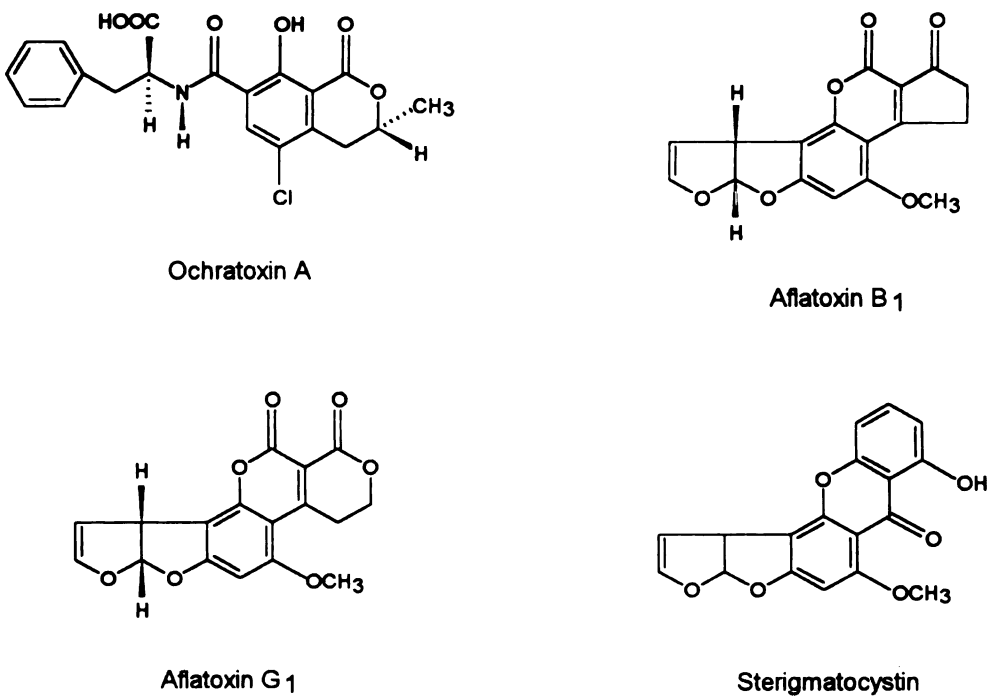

Figure 2. Structures of four important toxins produced by species of *Aspergillus*

Sources of Aflatoxin Production

The aflatoxins were initially identified in the 1960's as the causative agent of a deadly epidemic of turkey polts. This disease, known as Turkey "X" disease, claimed the lives of more than 100,000 turkeys. The vehicle of intoxication was found to be *A. flavus* contaminated feed (Ellis, 1991; Buchi and Rae, 1969). It was later discovered that aflatoxins are metabolic products of certain strains of *A. flavus*. Since then it has been

shown that all known strains of *A. parasiticus*, with one exception, also produce aflatoxins (CAST, 1989). *A. flavus* and *A. parasiticus* were generally accepted as the only aflatoxin producers until recently. A report by Kurtzman described a third species capable of producing aflatoxins-*A. nomius* (Kurtzman et al, 1987).

A. flavus spores are generally smooth, thin walled, and variable in shape - ranging from spherical to ellipsoid (Pitt, 1993). According to the ATCC, 29 isolates of *A. flavus* produce aflatoxins. It has also been reported that all of these isolates produce the B aflatoxins (ATCC, 1991). Six isolates (of the 144 reported in the 1991 ATCC catalogue) are reported to produce aflatoxins G_1 and G_2 as well. Strains of this species also produce toxins other than aflatoxins. One of the most important of these toxins, with respect to the food industry, is cyclopiazonic acid. The importance of cyclopiazonic acid is not due to its potency (which pales in comparison to that of aflatoxin) rather it stems from the fact that large quantities are produced after the fungus has colonized food products. Some strains of *A. flavus* have even been described in pathogenic infections causing acute aspergillosis (Bossche, 1987) in immunocompromised persons.

The strains of *A. parasiticus* differ morphologically from those of *A. flavus* in that *A. parasiticus* spores have thick, rough walls that are uniformly spherical (Pitt, 1993). There are fewer reported isolates (39), and all but one of the strains produce aflatoxins. The exception is a strain that produces o-methylsterigmatosystin (ATCC, 1991), a toxic precursor of aflatoxin. These strains produce B aflatoxins (B_1 and B_2) as well as the G aflatoxins (G_1 and G_2) - all four major aflatoxins. *A. parasiticus* also produces aspergillic acids and kojic acid which are not considered to be important toxins in the food industry. A search of the Medline, Agris, and Toxline literature databases did not indicate that *A.*

parasiticus has been implicated in pathogenic infections.

A. nomius is very difficult to distinguish from *A. flavus* because it too produces spores with smooth, thin walls of variable shape (Pitt, 1993). This species produces sclerotia which is the only morphological feature used to separate it from *A. flavus* (Pitt, 1992). *A. nomius*, like *A. parasiticus*, is able to produce the B and G aflatoxins and can serve as a fair indicator to distinguish *A. nomius* from *A. flavus*.

An indirect source of aflatoxins (via foods) is dairy cows. The use of aflatoxin contaminated crops as feed for cows results in the metabolism of aflatoxin M_1 (a hydroxylated form of AFB_1) by cows. This aflatoxin is found in the milk and meat that the cow produces. This contamination poses obvious potential health risks to human, most notably to infants consuming milk.

Prevalence of Aflatoxin Producing Strains

A. flavus, *A. parasiticus*, and *A. nomius* are considered ubiquitous in nature. That is they grow in many different environmental conditions and on various substrates. This is due to the fact that they are typical fungi and, therefore can grow over wide ranges of pH, temperature, and water activities (Cotty et al, 1994). The versatility of these aflatoxin producing fungi make them dangerous because they are able to grow on many food commodities. These commodities include cotton, cottonseed, corn, and peanuts. Growth of these fungi on these crops is opportunistic. This means that growth occurs on plants that are damaged in some way. Corn, for example, may be damaged before (by insects), during (by farming equipment), or after (handling practices/storage) harvest. As few as one damaged kernel can allow colonization by *Aspergillus* (Cotty et al, 1994).

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Subsequent dispersion of spores can occur at any time from growth in the field through processing, thereby increasing the potential for contamination. The colonizing fungus may or may not be an aflatoxin producer (as some strains of *A. flavus* do not produce aflatoxins) but the presence of the fungus can be sufficient for rejection of the crop.

Conditions for Fungal Growth and Aflatoxin Production

In general, strains of *A. flavus* and *A. parasiticus* grow optimally under similar conditions. The optimum growth temperature is between approximately 25°C and 30°C. Aflatoxin production takes place optimally around 29 to 30.5°C. The second most important growth factor is the relative humidity. For *A. flavus* and *A. parasiticus* a relative humidity between 88 and 95% results in optimal growth (Ellis, 1991; Bullerman, 1979).

Biological Effects of Aflatoxins

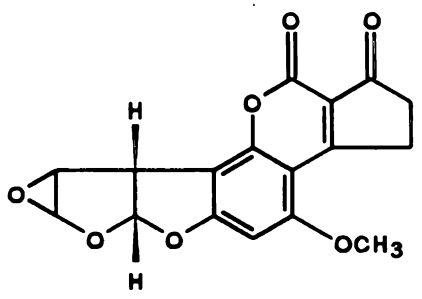
Many studies have been undertaken to determine the effects of aflatoxin poisoning on animals. It has been noted that while there are vast differences in susceptibility from species to species, those tested thus far are adversely affected by aflatoxins (Table2). The variations seen are caused by many factors including age, sex, nutritional status, health, and differences in activation and detoxification systems in the hosts cells.

Aflatoxins in their native form have low toxicity. After ingestion they are converted (activated) to a more toxic form and begin to exert their effects. The major method of conversion is by cytochrome P450 binding of an aflatoxin molecule (Massey et al, 1995; Gurtoo and Dave, 1975; Essigmann et al, 1982). This binding leads to the toxic

form of AFB_1 via conversion of the double bond in the furan ring to an epoxide (Figure 3).

Table 2. Sensitivity of Different Species to Aflatoxin B_1 fed Orally (adapted from Ellis et al, 1991)

Species	LD_{50} (mg/kg body weight)
Rabbit	0.3
Cat	0.55
Rainbow Trout	0.8
Dog	0.5 - 1.0
Guinea Pig	1.4 - 2.0
Baboon	2.0
Chicken	6.3
Rat (male)	5.5 - 7.2
Rat (female)	17.9
Mouse	9.0



AFB_1 8,9-epoxide

Figure 3. Structure of the highly reactive epoxide form of aflatoxin B_1.

The 8,9-epoxide is highly reactive and capable of covalently binding to macromolecules such as DNA and proteins (via the hydroxylated AFB_{2a}) (Eaton and Groopman, 1994; Dvorackova, 1990).

In the case of DNA binding, the reactive aflatoxin molecule shows an affinity for binding to the N^7 position of guanine bases (Massey et al, 1995; Harrison and Garner, 1991; Ball et al, 1990). The binding of DNA is postulated to be responsible for the mutagenic and carcinogenic effects of aflatoxins. Two methods of mutation exist after binding to DNA. The first method is disruption of replication and/or transcription due to the altered structure of the DNA (Hsieh, 1987). It is clear that disruption of binding by or processivity of polymerases (both DNA and RNA) are major concerns caused by structural alterations. The second method is that AFB_1-DNA adducts formed can result in GC→TA transversions during replication (Croy and Wogan, 1981). These transversions may lead to inactive protein molecules and possibly death of the cell depending on the importance of the altered gene. Another fate caused by transversion is the mutation of the *p53* tumor suppressor gene. Studies have shown that the carcinogenic effects of aflatoxins result from a specific mutation of codon 249 of the *p53* tumor suppressor gene (Massey, 1995; Bressac et al, 1991; Hsu et al, 1991).

Protein binding by aflatoxins also poses two threats. The most obvious of the two is that binding can cause conformational changes of proteins. By disrupting the folding of an enzyme the catalytic site of that enzyme may lose some or all of its activity. The second threat is the binding of aflatoxins by proteins which carry them to the nucleus. Obviously, this increases the possibilities of DNA binding (Massey et al, 1995).

Removal of the reactive epoxide form of aflatoxin is accomplished predominately by a glutathione S-transferase (GST) system. This system can be found in the cytosol and microsomes. GST catalyzes the conjugation of activated aflatoxins with reduced glutathione. This conjugation leads to the excretion of the activated aflatoxin (Neal and

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Green, 1983). The GSTs are composed of alpha, mu, pi, theta and microsomal classes. The activity of each class varies from species-to-species. Variation in activity of the GST system combined with the variation in activity of the cytochrome P450 activation system confer species differences in susceptibility to aflatoxins.

Aflatoxicosis

The disease caused by aflatoxin intoxication is aflatoxicosis. This disease can be broken down into three types based on its severity which is often determined by the levels of aflatoxins consumed. Acute and chronic aflatoxicoses result from consumption of high and intermediate levels of aflatoxins respectively. Acute aflatoxicosis results in death whereas chronic results in a reduction of growth rate and reproduction. Acute and chronic aflatoxicoses are considered subdivisions of primary aflatoxicosis. Both of the primary aflatoxicoses can be characterized by adverse effects seen in the liver, lungs, and sometimes the kidneys. Secondary aflatoxicosis results from low levels of aflatoxins. The major symptom of secondary aflatoxicosis is the weakening of the immune system. This leads to the inability of animals to defend themselves against invasion by pathogens and also hinders attempts at vaccination (Ellis et al, 1991; Piers et al, 1979). It is interesting to note that the effects of secondary aflatoxicosis may be cumulative. This would present the problem that no level of aflatoxin consumption is acceptable and that aflatoxins are a major health concern not only in third world countries but also developed countries.

The Aflatoxin Health Hazard

A major controversy has developed with respect to whether or not aflatoxins are, in fact, a health hazard to humans. The origins of this controversy can be identified as the

1) differences in susceptibility from one test animal to the next and 2) the high incidence of other health risks in areas along with aflatoxins. Obviously, no direct experiments have been carried out using human test subjects, leaving this question to be resolved with data from test animals. Differences between activation-detoxification systems have led to variable data from species to species (though the data do indicate that all test animals have shown adverse effects). Because of these differences it has been difficult to extrapolate the possible effects aflatoxins may have on humans. Outbreaks of liver cancer involving human populations, however, have helped to substantiate the circumstantial evidence obtained from test animals. Major outbreaks of hepatic cancer believed to be caused by aflatoxin consumption have occurred in India (Hsieh, 1986), China (Bressac et al, 1991), and Africa (Hsu et al, 1991). Opposing these data is the belief that primary liver cancer is the result of infection by hepatitis B virus (HBV) which is present in areas of high incidence of aflatoxin consumption (Stoloff, 1989).

Tests have shown that there are two major organs affected by aflatoxins: the liver, and the lungs. The liver is the site of the major AFB_1 activation system (cytochrome P450) and, therefore is the major organ affected by aflatoxin poisoning. Poisoning of test animals' livers has resulted in hepatic cancer.

An alternate route leading to lung aflatoxin exposure is via inhalation of dust and grains infested with aflatoxins which poses a threat to farm workers. Symptoms of aflatoxin poisoning of the lungs include cancerous growths and congestion (Moreau and Moss, 1979). The effects of aflatoxins on the lungs were shown following an incident in which several persons in Southeastern Asia died after consuming a diet containing aflatoxins. Aflatoxin-DNA adducts were revealed in the lung tissue of these victims

(Harrison and Garner, 1991). Other studies, using human cell lines, demonstrated the ability of lung cells to activate AFB_1 in the same manner as liver cells (cytochrome P450) (Autrup et al, 1979; Stoner et al, 1982; Mandal, 1987).

Methods of Controlling Aflatoxin Production

Many methods for controlling aflatoxins have been investigated. Among these methods are standard farming practices, biocompetition, and increased host defense mechanisms. Standard farming techniques entail thorough irrigation, careful harvesting practices and special environmental storage. In areas of persistant drought, adequate irrigation, let alone thorough, may be impossible. Even in countries where drought is not a problem thorough irrigation is deemed unfeasible due to the high cost of such a practice. Aside from the availability and cost problems of thorough irrigation there is the needs of different crops for different growth conditions. For example, the final stage (ripening) of peanut growth is characterized by drought stress. Obviously, irrigation of peanut crops during this stage would be detrimental to the final product. Careful harvesting practices are designed to reduce contamination by reducing the damage to and, consequently the susceptibility of crops. These practices do not ensure a reduction of contamination because it does not account for the damage of crops by insects. Another problem with careful handling practices is the cost of implementing them. This would be the result of slower harvesting and/or increased number of workers. Special environmental storage conditions describe the method of storing crops in silos that have controlled humidity and temperature. This method is capable of preventing both growth of and toxin production by fungi. The major problem with this method is once again money. To set up and

operate such a system costs far more than it may be worth. Also, this method is not able to stop growth by all strains at all times because of the ubiquity of the strains.

Biocompetition is a control method that has received much attention in recent years. This method involves seeding the soil of a field with a non-toxigenic strain early in the growing season. The hope is that this non-toxigenic strain will grow faster than the toxigenic strains thus excluding their growth. This method does not propose to introduce more fungal growth in the field, it simply aims to replace growth of toxigenic fungi with non-toxigenic fungi. Studies have shown (Cotty et al, 1990; Horn et al, 1994; Ehrlich, 1987) that this effect can be seen when applied to test plots. Dorner et al (1992) reported that the application of a competitive strain can decrease the total growth of other strains and total production of toxin. This "inhibitory" effect increased for as many as four years. At least two problems exist with this method of control. The first problem is the selection of strains for particular farming regions. The fungi that produce aflatoxins are ubiquitous in nature. Each strain, however, is suited to grow optimally in a particular set of environmental conditions (climate). This means that a strain which grows better than others in one climate may not be able to grow better than those same strains in another climate. In fact, it may be entirely possible that that same strain will not be able to grow at all in the second climate. In relation to this problem, fluctuations in climate (within a region) must be considered when selecting a biocompetitive strain. Changes in climate from year-to-year within the same region may have the same affect as described for differences between regions having different climates. Selection of a strain may not be a problem if the field is seeded with high numbers of the control strain at an early enough time in the growing season. The second problem encountered when using this method is

the fact that it does not reduce the contamination of crops by fungal growth. Inherently, this method requires contamination. Because a crop can be rejected on the basis of fungal contamination this method does not eliminate one of the main concerns generally ascribed to aflatoxin production - economic.

Increased host defense mechanisms refers to the ability of a plant to defend itself against colonization and/or production of toxin by fungi. Chemicals have been identified that inhibit growth and/or toxin production. By use of molecular techniques the effects of inhibitory chemicals can be characterized at a genetic level. These methods may show that an inhibitor acts at the biochemical pathway which is responsible for the production of aflatoxins. Alternatively, they could show that an inhibitor may act as a fungicide thus preventing growth of the fungus. If the production of these chemicals can be incorporated as a host plant defense mechanism then it is possible that the colonization by or production of toxin can be reduced. Problems that exist with this strategy are the identification of inhibitors, the number of possible crops that would need modification, and the reception of this method by the public. The first problem can be addressed by trying to find inhibitors produced by the crop itself - which would be preferred. For example, peanuts produce 5,7-dimethoxyisoflavone. When extracts of this compound are applied to developing plants in the presence of toxigenic *A. flavus* spores growth of the fungus is greatly reduced (Turner et al, 1975). Increasing the production of this inhibitor by modifying its regulation may adequately protect peanut crops. The second problem may be resolved by the possible solution of the first. If each crop naturally produces an inhibitor of either growth or toxin production, the modification of each crop may be similar for many of the crops. The final problem is typically defined as a mistrust of genetics by the public. Past attempts

to market products that have been associated with genetic modification, such as the frost free strawberry, have met much resistance. A way around this problem may be breeding natural producers of inhibitors to express those inhibitors at higher levels, this solution is dependent on the resolution of problems one and two. Nevertheless, the marketing of such products will be highly dependant on education of the non-scientific community. This education must start with regulators to ensure that reasonable (as opposed to excessive) precautions/warnings are assigned to these products. In this way a paranoia by the general public can be avoided to some extent. Ultimately, the public will decide on the feasibility of modified products. It is easy to confuse persons on complicated issues. Terms such as "antisense RNA" and "gene inactivation by deletion" are not likely to become household jargon any time soon. People will always be able to introduce some doubt, confusion or even fear of these kinds of products. The advent of the flavor savor tomato was a step in the right direction but to continue that progress a spokesperson or committee aimed at educating the public about new products should be formed.

Short Term Goal

Clearly, the long term goal of aflatoxin research is to eliminate aflatoxins from the food chain. The work presented in this paper takes a molecular approach toward achieving this goal. The main focus of this work was to characterize growth, aflatoxin production, and the presence of aflatoxin genes and transcripts by and in several species of *Aspergillus*. The strains selected for this study include one *A. parasiticus*, two *A. flavus* aflatoxin producers, two *A. flavus* suspected aflatoxin non-producers, one *A. sojae* suspected aflatoxin non-producer, and one *A. oryzae* suspected aflatoxin non-producer.

At the beginning of this project, it was hoped that characterizing the differences between these strains might lead to clues as to why some strains produce aflatoxins whereas others do not (even if they have the aflatoxin genes). With this kind of information it may be possible to genetically modify toxigenic strains in such a way that they behave like non-toxigenic strains. It was also hoped that we could determine if industrial strains (represented by *A. sojae* and *A. oryzae*) also possess the biosynthetic pathway for aflatoxin production. At the moment it is unknown why some strains produce aflatoxins whereas others do not (this referring to *A. flavus* more than *A. parasiticus* or *A. nomius*). It is thought that the inability of some strains to produce aflatoxins is brought about by mutations to regulators of the pathway rather than enzymes of the pathway. Several studies have shown that disrupting enzymes results in an accumulation of toxic aflatoxin precursors. Disruption of genes such as *nor*-1 (Trail et al, 1994), *ver*-1 (Skory et al, 1992), and *omt*-1 (Yu et al, 1993) result in an accumulation of norsolorinic acid, versicolorin A, and sterigmatocystin, respectively. Three genes *afl*-R (Payne et al, 1993), *fas*-1A (Mahanti et al, 1996), and *pks*A (Chang et al, 1995) have been disrupted without an accumulation of precursors. It has been hypothesized that the *afl*-R is a regulator though its mechanism of action has not been shown.

Materials and Methods

Strains and Culture Conditions

Growth conditions for dry weight, aflatoxin and RNA extraction

Seven strains of *Aspergillus* were used in this study. *A. parasiticus* NRRL 5862 (SU-1), *A. flavus* SRRC 1059 (1059), and *A. flavus* SRRC 1273 (1273) comprised the aflatoxin producing strains. *A. flavus* SRRC 2112 (2112), *A. flavus* SRRC 2115 (2115), *A. oryzae* ATCC 14895 (AO), and *A. sojae* ATCC 42251 (AS) were the suspected aflatoxin non-producers.

Cultures of each strain were grown in duplicate by inoculating 100ml of Reddy's medium (in 250ml flasks)(Reddy et al, 1971) with 10^7 spores per flask. Each pair of cultures was allowed to grow in the dark at 29°C while stationary or shaking at 150 rpm (in a New Brunswick G-25 floor model shaker). Two time periods of growth were selected, 48 and 72 hours, based on the fact that the aflatoxin gene transcripts are not easily detectable before 48 hours and begin to degrade at some time between 72 and 84 hours (Skory et al, 1993). It should be noted that the aflatoxin gene transcripts may be produced earlier than 48 hours but insufficient cell mass (for RNA extraction) was available before that time in the stationary cultures. At the end of the growth period the contents of the flasks were vacuum filtered using a Buchner funnel and mira cloth. For every flask, five ml of growth medium were removed and stored at -20°C prior to ELISA analysis for aflatoxins. From the first flask of each pair the mycelia were scraped from the mira cloth and dried under vacuum at 80°C for one hour. The mycelia from the second

flask were also scraped off the mira cloth but this material was halved for RNA extraction and aflatoxin extraction (for ELISA analysis).

Growth conditions for DNA extraction

Approximately 10^7 spores were inoculated into 100ml of yeast extract (20g/l)-sucrose (60g/l) medium (YES) in a 250ml flask. The flasks were incubated for 48 hours in the dark at 29°C while shaking at 150 rpm.

Spore preparation procedure

One hundred fifty ml of Potato Dextrose Agar (PDA) were solidified in a one liter Brockway glass bottle and inoculated by streaking spores across the agar surface with a sterile loop. The cultures were allowed to grow for 10 days at 29°C in darkness. The spores were harvested by aseptically pouring approximately 50 - 100ml of sterile water into the bottle followed by the addition of a sterile magnetic stir bar (Baxter). The bottle was then placed on a NuovaII stir plate to remove the growth from the agar. The contents (excluding the stir bar) of the bottle were poured into a 60ml Tuberculin syringe (Becton Dickinson & Co.) containing a plug of sterile glass wool (Pyrex). The growth suspension was allowed to filter through the syringe and glass wool (the spores passed through the glass wool but the mycelial fragments were retained) into a 250ml GSA bottle (Sorvall). At the same time another 50 ml of sterile water was added to the bottle to remove any growth that may still have been in the bottle. This suspension was also added to the syringe. The contents of the syringe were then compressed with the plunger to remove as much liquid (presumably containing spores) as possible. The GSA bottle was then centrifuged in a RC2 (Sorvall) centrifuge at 5856xg for 10 minutes at 4°C. The supernatant was carefully poured off and the pellet was suspended in approximately five

ml of sterile 20% glycerol (Boehringer Mannheim Biochemical). Ten µl of the sample was used to determine the concentration of the suspension using a hemocytometer and bright field microscope (American Optical). The spore suspension was finally distributed to 1.5ml micro centrifuge tubes and stored at -80°C until used.

Extraction of Genomic DNA

Extraction of genomic DNA was performed by grinding mycelia in a mortar using a pestle and liquid nitrogen, followed by two phenol-chloroform extractions and ethanol precipitation (Ausubel et al, 1987; Skory, 1992).

Southern Hybridization Analysis

Genomic DNA was cut with restriction enzymes (Boehringer Mannheim Biochemical) and electrophoresed on a 1% agarose gel for four hours at 80 volts. Following electrophoresis the DNA was transferred to Nytran (S&S) nylon membrane using the method of Maniatis (1989). Southern hybridization analysis was performed at 42°C for 16 hours (in a Robbins Hybridization Incubator 310) using the labeled (^{32}P) *nor*-1 (1.5-kb *Eco*RI/*Cla*I fragment of pNA17) and *ver*-1A (1.8 -kb *Eco*RI/*Sal*I or 0.7-kb *Eco*RI/*Bam*HI fragment of pBSV2) genes from *A. parasiticus* NRRL 5862 as probes. The membranes were washed twice for 15 minutes with 2XSSC, 0.1%SDS at room temperature followed by a one hour wash at 65°C using a 0.1XSSC, 0.1% SDS buffer. Autoradiography was performed with Kodak XAR-5 film at -80°C.

Extraction of Aflatoxins from Mycelia

Aflatoxins were extracted from mycelia by adding five ml of 100% methanol to half the mycelial contents of one growth flask. The methanol-mycelia samples were agitated briefly and then allowed to stand for at least one hour prior to removing the methanol which was then stored at -20°C until needed for thin layer chromotography (TLC) or ELISA analysis for aflatoxins (Dvorackova, 1990).

ELISA Analysis for Aflatoxins

Direct competitive ELISA analysis was performed by the method of Pestka (1988). The polyclonal antibody, 5C11, (directed against AFB_1) and the aflatoxin-peroxidase conjugate, 80B, were kindly provided by Dr. James J. Pestka. A 1:500 dilution of the antibody as well as the aflatoxin-peroxidase conjugate were used. Aflatoxin standards were prepared with an aflatoxin B_1, B_2 mixture (Sigma Chemical Co., St. Louis, MO) which was dissolved in 100% methanol. Some of this stock was diluted to one ng/µl for preparation of the standards (0.5, 1.0, 5.0, 10, 50, and 100 ng/ml) used in the procedure. The absorbance of each well of the microtiter plates was read using a Vmax spectrophotometer (Molecular Devices) using Softmax Software running on an IBM model Z50 computer.

Extraction of Total RNA

Total RNA was extracted from approximately 1.5 g of wet mycelia (if available) by the hot phenol method of Maramatsu (1973).

Northern Hybridization Analysis

Forty μg of total RNA were electrophoresed on a denaturing formaldehyde agarose gel (0.8%) at 75 volts for 3.5 hours. The RNA was then transferred to Nytran plus nylon membrane (S&S) by the method of Maniatis (1989). The membranes were then probed for 16 hours at 42°C using the labeled (^{32}P) DNA fragments in Table 3. The membranes were then washed. Autoradiography was performed with Kodak XAR-5 film at -80°C.

Table 3. Gene fragments used in Northern Hybridization analysis

Gene	Plasmid	Restriction enzyme(s)	Size of fragment
afl-R	*Sac*I fragment	*Eco*RI	0.5 -kb
β-tubulin	pAPBENK	*Sac*I - *Kpn*I	1.0 -kb
nor-1	pNA-17	*Hin*dIII - *Cla*I	1.0 -kb
*pks*A	pAPNVES4.3	*Eco*RI - *Sac*I	4.3 -kb
*pyr*G	pPG3J	*Pst*I	1.2 -kb
ver-1	2.1-kbver	*Bam*HI - *Eco*RI	0.7 -kb

Thin Layer Chromatography (TLC)

Thin layer chromatography was carried out to resolve aflatoxins for qualitative purposes. Whatman Silica plates (cat#05713264) were spotted with 10μL of sample and 250ng of aflatoxin B_1 standard (Sigma). The plates were then placed in a chamber containing chloroform (J.T. Baker) and acetone (Mallinckrodt) at a ratio of 95:5. The samples were resolved for 45 minutes. The plates were removed and allowed to dry, and then viewed under long wave UV light. The appearance of blue bands which comigrated

with standard indicated the presence aflatoxin B_1.

Precautions When Handling Aflatoxins

Because aflatoxins are potent carcinogens the handling and disposal of them are must be carefully executed to avoid exposure. Whenever a container with aflatoxins (whether in liquids or cells) was handled a laboratory coat and gloves were worn. Grinding of mycelia (during DNA and RNA extractions) results in particulate "mycelial" dust, therefore, this procedure was carried out in a biological hood and a mask was worn. Disposal of aflatoxins involved chemical inactivation. This was accomplished by treating all aflatoxin contaminated containers, growth media, and cells with a 5% bleach solution (final concentration) for at least a half hour. Acetone was then added to a concentration of 5% to stabilize the reaction.

Results

Southern hybridization analysis

Southern hybridization analysis using high stringency washing conditions was conducted on genomic DNA extracted from *A. parasiticus* SU-1 (wild type) by Skory (1992). Several restriction enzymes were used to cut the DNA before being probed with (^{32}P) *ver*-1A and (^{32}P) *nor*-1 DNA probes. Nine of the enzymes used (known not to cut within the *ver*-1A) produced two DNA fragments capable of hybridizing the *ver*-1A DNA probe. The *Bam*HI digest produced four bands even though it was known only to cut once within *ver*-1A. These data led to the identification of a second copy of the *ver*-1A gene in *A. parasiticus* - called *ver*-1B. Nucleotide sequence analysis of *ver*-1B demonstrated that it is 93% identical to the *ver*-1A at the nucleotide level (Liang et al, 1994). Furthermore, a translational stop codon was reported early in the *ver*-1B coding sequence prompting the belief that this gene produces a truncated protein with little or no function. Hybridization of these same digests with the *nor*-1 probe indicated that only one copy of *nor*-1 is present in *A. parasiticus* based on the fact that no unexpected bands were identified (Skory, 1992).

Southern hybridization analysis using was conducted on *Bam*HI digested genomic DNA of several *Aspergillus* spp. (both aflatoxin producers and aflatoxin non-producers) in order to determine if genes similar to *ver*-1A and *nor*-1 could be identified in these species. Only *A. versicolor* and *A. tamarii* failed to hybridize with either probe whereas *A. nidulans* hybridized with the *ver*-1A probe but not the *nor*-1 probe under high stringency

conditions. For the rest of the species investigated the probes hybridized to only one DNA fragment each suggesting that only one copy of a gene similar to *ver*-1A and one similar to *nor*-1 is present (Skory, 1992). These data suggested that the aflatoxin genes are not present soley in aflatoxin producing strains. The aflatoxin non-producing strains included three *A. sojae* and one *A. oryzae*. These findings are of particular interest due to the industrial use of some strains of these species in fermentations.

Rayard Thomas, in our laboratory, conducted further Southern hybridization analyses on *Hin*dIII restricted genomic DNA isolated from the following *A. flavus* strains: SRRC 284 (afl^-), SRRC 285 (afl^-), SRRC 2112 (afl^-), SRRC 2115 (afl^-), SRRC 1000A (afl^+), SRRC 1059 (afl^+), and SRRC 1273 (afl^+). Using a 0.7-kb (*Eco*RI-*Bam*HI) *ver*-1A probe, Thomas was able to show that all but one of the strains contain a single *ver*-1A-like sequence of approximately 15.4-kb in size (unpublished data). SRRC 2112, however, produced two bands of equal intensity corresponding to approximately 15.8 and 13.8-kb (Figure 4). These findings suggested that 2112 (like SU-1) may contain two copies of the *ver*-1A like sequence. One theory as to why nearly all *A. parasiticus* strains produce aflatoxins is due to duplications of genes in the *A. parasiticus* aflatoxin pathway. In theory, this would make *A. parasiticus* more resistant to mutations than *A. flavus* due to redundancy. SRRC 2112 is a suspected aflatoxin non-producer. If it does have multiple copies of the *ver*-1A sequence this may suggest that non-producers have gene duplications similar to the *A. parasiticus* strains. If this is so, it would be more likely that a mutation to a regulator has caused the inactivation of the aflatoxin pathway rather than mutations in a number of the genes. *A. parasiticus* has multiple copies of the *afl*-R (Payne et al, 1993) meaning that mutations to both copies would need to occur to eliminate its function. To

determine if suspected aflatoxin non-producers are deficient in the *afl*-R product it would be helpful to determine the number of copies present in the genome.

Using a 1.7-kb (*Bgl*II-*Sph*I) *nor*-1 probe two major DNA fragments were detected in all *A. flavus* strains tested (Thomas). These fragments were approximately 3.4 and 3.2-kb in size (Figure 4). Additional weaker signals hybridized to the *nor*-1 probe in genomic DNA of strains SRRC 284, 2112, 1000A, and 1059 at 5.4 and 6.8-kb. Finally, 2112 genomic DNA hybridized to the *nor*-1 probe also at 9.4-kb and 4.1-kb. These results indicate that there is one gene in all strains which appears to be nearly identical to *nor*-1 from SU-1. However, a variable number of less related genes may be present in strains SRRC 284, 2112, 1000A, and 1059.

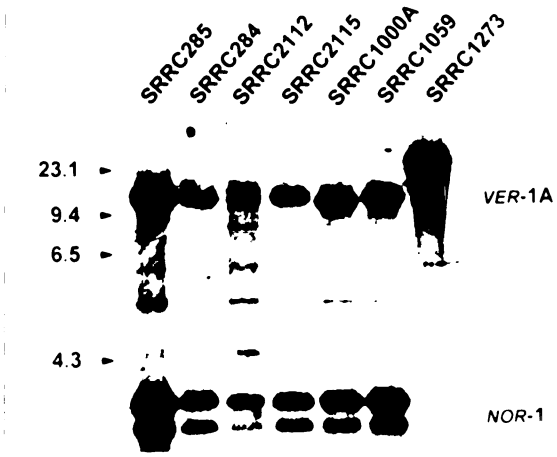


Figure 4. Southern hybridization of *Aspergillus flavus* strains with *nor*-1 and *ver*-1 probes. Southern analysis was performed once on SRRC 284, 285, 2112 and 2115 (aflatoxin non-producers); and SRRC1000A, 1059, and 1273 (aflatoxin producers).

Due to the fact that Skory and Thomas both used incompletely cut (*Hin*dIII) 1273 genomic DNA samples when performing their Southern analyses it was necessary to perform another Southern analysis of this strain. The genomic DNA for 1273, 2112, and SU-1 was prepared and fractionated as described in the materials and methods. Using the *ver*-1A and *nor*-1 probes (listed in Table 3) it was found that only a single signal was present for each probe in all three strains (Figure 5). For the *ver*-1A probe this DNA fragment was significantly smaller (approximately 10.5-kb) than that observed by Skory and Thomas. Because the signals observed for 1273 and SU-1 were the same size, however, the differences between these sizes and those seen by Skory and Thomas may be due to differnces in electrophoresis conditions and/or the transfer procedure.

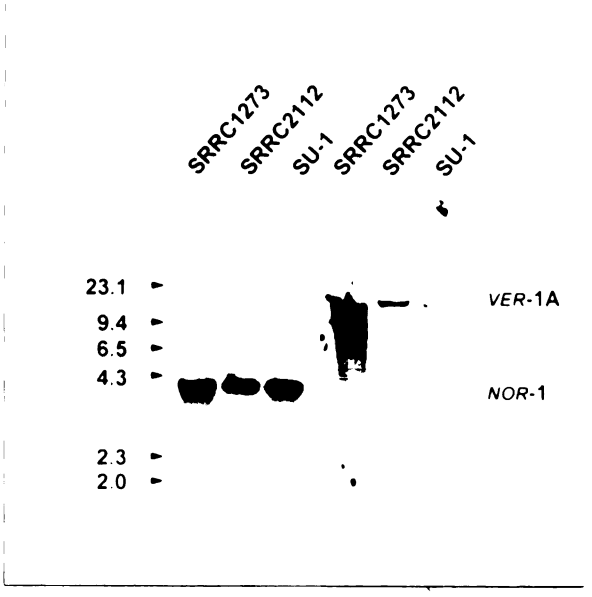


Figure 5. Southern hybridization of 1273, 2112, and SU-1 with A) *nor*-1 and B) *ver*-1 probes.

The *nor*-1 probe revealed one signal at approximately 3.9-kb which is similar to the sizes reported by Skory and Thomas. The reason that there are two bands reported by Skory and Thomas is that their probes contained a *Hin*dIII site, whereas the *nor*-1 probe used to generate these data did not.

Dry weight measurements

Dry weights of the mycelia from each of the strains were determined in order to calculate the amounts of aflatoxins produced per milligram of mycelium (Table 4). The growth of the stationary cultures was very slow with total cell mass never reaching one gram. The shake cultures showed higher growth rates compared to those reported by Trail et al (1995).

Table 4. Dry mycelial weights (grams) of *Aspergillus* spp. grown on Reddy medium to 48 and 72 hours in shake and stationary cultures. Weights are based on half a mycelial pad from one of the replicate flasks.

	Stationary		Shake	
	48 hours	72 hours	48 hours	72 hours
SU-1	0.10g	0.12g	1.62g	2.19g
2112	0.15g	0.10g	1.11g	1.69g
2115	0.24g	0.14g	1.28g	1.58g
1059	0.21g	0.36g	2.21g	2.44g
1273	0.15g	0.28g	2.33g	2.78g
AO	0.22g	0.19g	1.16g	2.25g
AS	0.10g	0.11g	1.48g	2.53g

The decreased growth in stationary cultures (compared to shake cultures) may be due to

decreased exposure to oxygen during germination and subsequent growth. Preliminary data suggest that the effect is caused by decreased germination rate. Zhou (personal communication) has indicated that shaking a culture for 24 hours then allowing it to grow as a stationary culture results in increased growth at later time points. The 24 hour time point is significant in his study because there does not appear to be any noticeable growth at that time.

ELISA analysis

ELISA was performed in order to determine the amounts of aflatoxins per milligram of mycelial dry weight being produced by the seven *Aspergillus* strains and the proportion of toxin production from mycelia and medium for the stationary and shake cultures respectively (Tables 5 and 6). A comparison of toxin levels found in the growth medium demonstrated that toxin production was generally reproducible in duplicate flasks. No aflatoxins were found in the media nor the mycelia from any of the suspected aflatoxin non-producers.

Table 5. Analysis of aflatoxin B_1 production from stationary cultures. Replicates 1 and 2 indicate aflatoxin levels found in the media of replicate growth flasks. Only one extraction of mycelia was performed (therefore no replicate is shown).

Stationary 48 hours:						
	Toxin in Replicate 1	Toxin in Replicate 2	Toxin in Mycelia	Total Toxin	Dry Weight	Toxin/mg Mycelia
SU-1	86.2µg	90.6µg	5.0µg	93.4µg	0.10g	930ng/mg
2112	ND	ND	ND	-	0.15g	-
2115	ND	ND	ND	-	0.24g	-

Table 5 (cont'd)

AS	ND	ND	ND	-	0.10g	-
AO	ND	ND	ND	-	0.22g	-
1059	101.9µg	75.6µg	5.2µg	93.9µg	0.21g	440ng/mg
1273	94.0µg	91.7µg	6.8µg	99.7µg	0.15g	660ng/mg
Stationary 72 hours:						
SU-1	80.6µg	72.4µg	12.7µg	89.2µg	0.12g	740ng/mg
2112	ND	ND	ND	-	0.10g	-
2115	ND	ND	ND	-	0.14g	-
AS	ND	ND	ND	-	0.11g	-
AO	ND	ND	ND	-	0.19g	-
1059	57.4µg	94.0µg	21.1µg	96.8µg	0.36g	270ng/mg
1273	79.7µg	60.5µg	9.99µg	80.1µg	0.28g	290ng/mg

ND - none detected

Table 6. Analysis of aflatoxin B_1 production from shake cultures. Replicates 1 and 2 indicate aflatoxin levels found in the media of replicate growth flasks. Only one extraction of mycelia was performed (therefore no replicate is shown).

Shake 48 hours:						
	Toxin in Replicate 1	Toxin in Replicate 2	Toxin in Mycelia	Total Toxin	Dry Weight	Toxin/mg Mycelia
SU-1	56.0µg	52.7µg	4.50µg	58.9µg	1.62g	36ng/mg
2112	ND	ND	ND	-	1.11g	-
2115	ND	ND	ND	-	1.28g	-
AS	ND	ND	ND	-	1.48g	-
AO	ND	ND	ND	-	1.16g	-
1059	53.4µg	46.8µg	5.52µg	55.6µg	2.21g	25ng/mg
1273	56.0µg	46.5µg	5.52µg	56.8µg	2.33g	24ng/mg

Table 6 (cont'd).

Shake 72 hours:						
SU-1	121.9µg	123.8µg	12.5µg	135µg	2.19g	62ng/mg
2112	ND	ND	ND	-	1.69g	-
2115	ND	ND	ND	-	1.58g	-
AS	ND	ND	ND	-	2.53g	-
AO	ND	ND	ND	-	2.25g	-
1059	110.0µg	128.2µg	12.6µg	132µg	2.44g	54ng/mg
1273	131.1µg	121.3µg	13.0µg	139µg	2.78g	50ng/mg

ND - none detected

The 48 and 72 hour stationary cultures produced roughly the same amount of AFB_1 which was nearly twice the amount produced by the 48 hour shake culture. The 72 hour shake culture, however, produced nearly 30% more AFB_1 than the 48 or 72 hour stationary cultures. In terms of AFB_1 production per milligram of dry weight, SU-1 produced more toxin than 1059 and 1273 at either time point in stationary or shake culture. For example, SU-1 produced 13% more toxin than 1059 at the 72 hour time point in shake. The equivalent stationary culture comparison revealed a difference of 64%. It can also be seen that the stationary cultures produced more toxin per milligram of dry weight than the shake cultures. For example, in the case of SU-1 at 48 hours, the stationary culture produced nearly 30 times more toxin per milligram of mycelia. Because stationary cultures better mimic the conditions the fungi are likely to encounter in the field, these cultures may give a more accurate account of aflatoxin production than shake cultures. If aflatoxin production is regulated at the level of transcription (Skory, 1993) then using stationary cultures may be more appropriate for studies of regulation. Finally, it appears that the mycelia accumulate aflatoxin as the culture grows but this quantity is small

compared to the accumulation of aflatoxin in the medium. Also, there is no difference between the amount of toxin retained by mycelia from shake and stationary cultures. These data indicate that there is some mechanism that eliminates aflatoxin from the fungal cells although it is not clear whether this mechanism is active or passive.

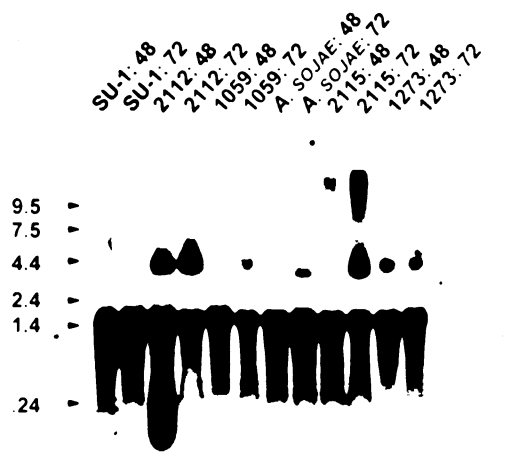
Northern hybridization analysis

Northern hybridization analyses were performed on total RNA extracted from each strain grown for 48 or 72 hours under shake or stationary culture conditions to determine if the genes detected using Southern analysis (*nor*-1 and *ver*-1A) were being expressed. Six probes (listed in Table 3) were used. Unfortunately, the *afl*-R probe only hybridized to ribosomal RNA in all of the samples (data not shown). The β-tubulin and *pyr*G gene probes were used in order to demonstrate that the amounts of RNA loaded in each lane was consistent (Figure 6 and Figure 7 respectively). The size of the RNA transcript detected by the β-tubulin probe for the shake cultures was approximately 1.3-kb (Figure 6a). This is consistent with the transcript size reported by Trail et al (1995) for cells grown on Reddy medium. In this same study in cells grown in YES medium no transcript could be detected at any time after 24 hours (up to 72 hours). Also, a 3.5-kb transcript was detected in some of the shake cultures (2112 at 48 and 72 hours, 1059 at 72 hours, AS at 72 hours, 2115 at 72 hours, and 1273 at 48 and 72 hours) that was not detected in the previous study (Trail). There did not seem to be a pattern to which strains did or did not produce this transcript. For example, some aflatoxin producers expressed this transcript early and late (1273), whereas others only produced it at one time point (1059), and still others not at all (SU-1). The suspected aflatoxin non-producers were also in this category. The unpredictable appearance of the 3.5-kb transcript may be related to

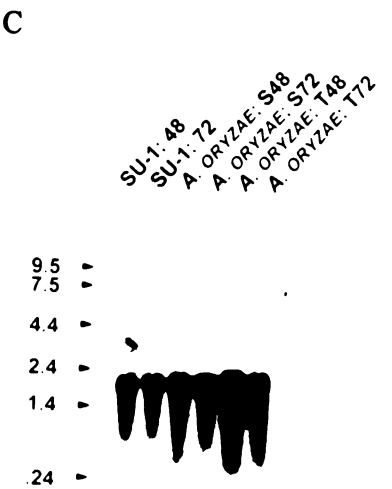
Figure 6. Northern hybridization of A) shake cultures, B) stationary cultures, and C) AO shake and stationary cultures using the β-tubulin probe.

Figure 6.

A



C



C) A0

B

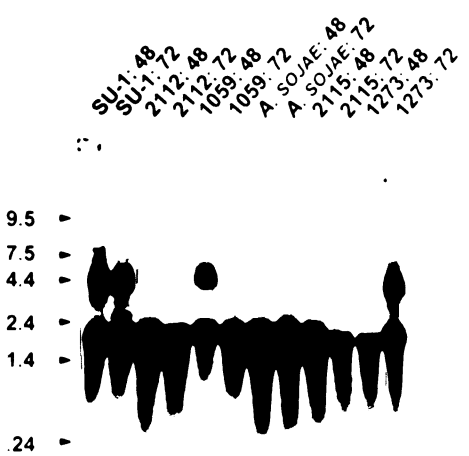
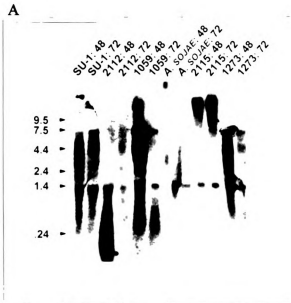
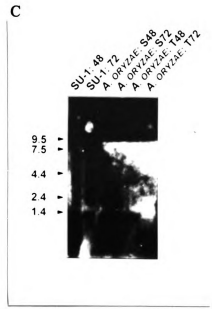
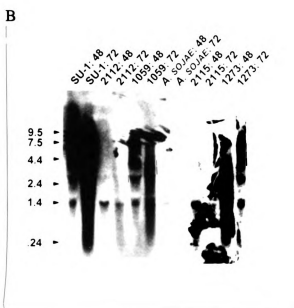


Figure 7. Northern hybridization of A) shake cultures, B) stationary cultures, and C) AO shake and stationary cultures using the *pyr*G probe.

Figure 7.


A
SU-1, 48
SU-1, 72
2112, 48
2112, 72
1059, 48
1059, 72
A. SOJAE, 48
A. SOJAE, 72
2115, 48
2115, 72
1273, 48
1273, 72
9.5
7.5
4.4
2.4
1.4
.24


C
SU-1, 48
SU-1, 72
A. ORYZAE, S48
A. ORYZAE, S72
A. ORYZAE, T48
A. ORYZAE, T72
9.5
7.5
4.4
2.4
1.4


B
SU-1, 48
SU-1, 72
2112, 48
2112, 72
1059, 48
1059, 72
A. SOJAE, 48
A. SOJAE, 72
2115, 48
2115, 72
1273, 48
1273, 72
9.5
7.5
4.4
2.4
1.4
.24

differences in the way these species regulate transcription of this particular transcript but do not reflect differences in growth of RNA in each lane. The stationary cultures differed from the shake in that they produced a primary signal at 1.7-kb (Figure 6b). The stationary cultures produced a secondary band (4.1-kb) that was also bigger than its shake culture counterpart. The absence of a pattern for the production of this secondary signal exists in these cultures too. Strangely, many of the strains that did not produce any of the secondary transcript in shake culture did produce it in stationary culture (ie. SU-1 at 48 and 72 hours). If the speculation that these secondary band differences are the result of differences in regulation then it may also be true that the conditions that the cultures are grown under may greatly influence transcription. The implications of this are that stationary cultures (which more closely mimic the conditions the fungi encounter on host plants) may be more appropriate for the study of the mechanisms controlling aflatoxin production.

RNA isolated from shake and stationary cultures was also analyzed with a *pyr*G probe (Figure 7). The size of the *pyr*G transcript in shake culture was measured at 1.4-kb which is consistent with the results seen by Trail. Likewise, the size of the signal produced by the stationary cultures (1.2-kb) was reasonably close to the results seen by Trail though her results did not reveal a signal after 40 hours. There may be no significant difference between the sizes because the discrepancy may be an artifact of measuring or gel handling. Once again, this main band shows that the loading of RNA from lane-to-lane was consistent.

Northern analyses also were performed on RNA isolated from each of the strains grown for 48 or 72 hours in shake or stationary cultures to identify transcripts that

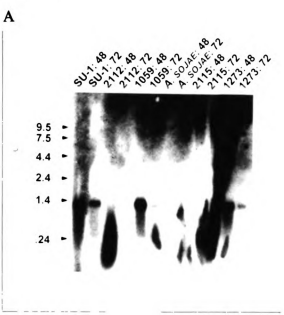
hybridized to a *nor*-1 probe (Figure 8a, b, and c). The shake cultures of the aflatoxin producers contained transcripts of approximately 1.0-kb whcih hybridized to *nor*-1. SU-1 appeared to accumulate the most transcript followed by 1059 then 1273. Higher levels of transcript were detected at 72 hours as compared to 48 hours. These results are consistent with those observed on SU-1 (Trail). The suspected aflatoxin non-producers did not produce detectable levels of transcript. This indicates that the *nor*-1 genes tentatively identified by Southern hybridization analyses are not actively transcribed in these strains. The suspected aflatoxin non-producer 2112 also shows a widely dispersed signal at approximately 0.2-kb with the *nor*-1 probe. This signal also occurred under these culture conditions for every probe used suggesting that an impurity in the RNA might account for this non-specific hybridization. Several independent extractions of RNA from this strain in shake culture showed this "blob" during northern hybridization analyses. This combined with the fact that this "blob" did not appear in stationary culture suggested that the problem was, in fact, not caused by impure RNA. Based on the fact that this signal only occurs at the 48 hour time point suggests that this it is an RNA degradation product that is completely degraded by the 72 hour time point. This theory could be tested by conducting northern analyses at earlier time points and time points between 48 and 72 hours. The presence of this "blob" is still a mystery that, because it appears to be non-specific, may not warrant further investigation from the standpoint of aflatoxin biosynthesis. Figure 8b shows that the same pattern of hybridization occurred with the stationary cultures except that the 0.2-kb hybridization did not appear.

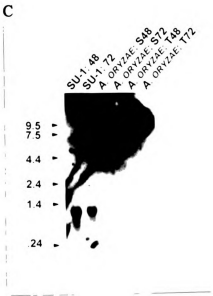
Northern hybridization analysis was also used to show the pattern of expression for the *ver*-1 transcript in the seven strains for shake cultures, stationary cultures, and AO

samples (shake and stationary) (Figure 9a, b, and c respectively). In shake culture only the aflatoxin producers had transcripts similar in size to that reported for the *ver*-1 (1.0-kb). Higher transcript levels were observed at the early time points (48hours). Again, SU-1 produced more transcript than 1059 and 1273 (second and third respectively). Once again a "blob" appeared at approximately 0.2-kb for 2112 at the 48 hour time point. Unexpectedly, a similar signal was detected at the 72 hour time point in 2112, AS, and 1273. These "blobs" did not occur with any probes other than the *ver*-1A nor did they occur in stationary cultures. This combined with the fact that it is a late time point (*ver*-1A being a late gene in the pathway) may suggest an altered form of transcription (potentially the regulation) involving *ver*-1A or simply a non-stable transcript. The *ver*-1A hybridizations using the stationary cultures revealed a different pattern. The major signal remained at approximately 1.0-kb but the 72 hour time point appeared to have more transcript present. This shift from more accumulation at the early time point to more accumulation at the late time point may indicate that shake cultures begin to produce aflatoxins earlier than the stationary cultures. A secondary signal was also produced at roughly 4.5-kb. This secondary signal appeared to be present only when the primary band was strong. For example, SU-1 produced the most intense band at 1.0-kb and had the most intense secondary band. The 1059 samples had much weaker primary signals and barely detectable (if at all) secondary bands. Even more extreme, the 1273 samples produced barely detectable primary bands and no signal was present in the area around 4.5-kb.

Figure 8. Northern hybridization of A) shake cultures, B) stationary cultures, and C) AO shake and stationary cultures using the *nor*-1 probe.

Figure 8.


A
SU-1 48
SU-1 72
2112 48
2112 72
1059 48
1059 72
A. SOJAE 48
A. SOJAE 72
2115 48
2115 72
1273 48
1273 72
9.5
7.5
4.4
2.4
1.4
.24


C
SU-1 48
SU-1 72
A. ORYZAE S48
A. ORYZAE S72
A. ORYZAE T48
A. ORYZAE T72
9.5
7.5
4.4
2.4
1.4
.24

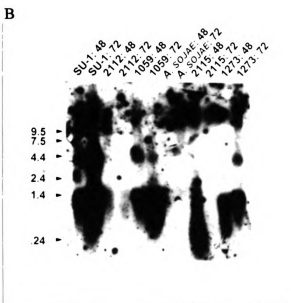
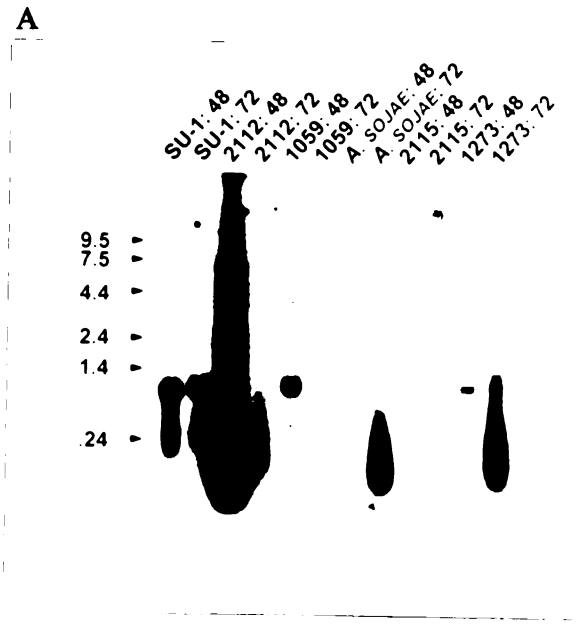
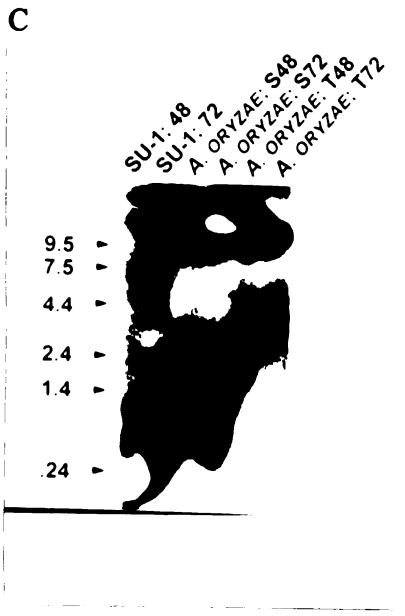
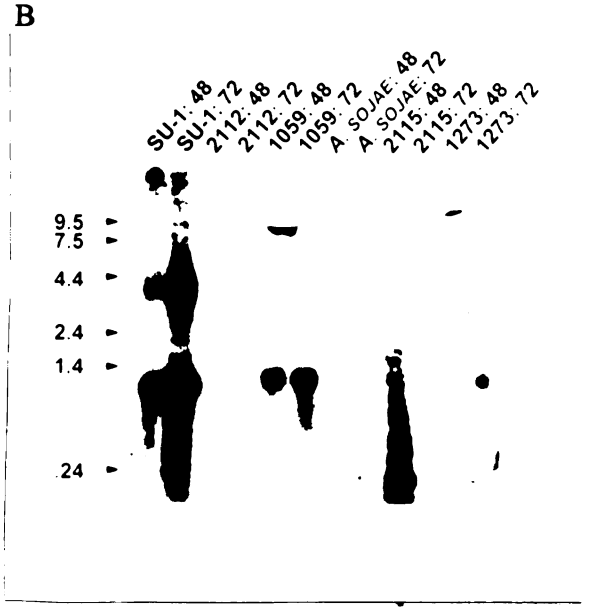

B
SU-1 48
SU-1 72
2112 48
2112 72
1059 48
1059 72
A. SOJAE 48
A. SOJAE 72
2115 48
2115 72
1273 48
1273 72
9.5
7.5
4.4
2.4
1.4
.24

Figure 9. Northern hybridization of A) shake cultures, B) stationary cultures, and C) AO shake and stationary cultures using the *ver*-1 probe.

Figure 9.


A
SU-1: 48
SU-1: 72
2112: 48
2112: 72
1059: 48
1059: 72
A. SOJAE: 48
A. SOJAE: 72
2115: 48
2115: 72
1273: 48
1273: 72
9.5
7.5
4.4
2.4
1.4
.24


C
SU-1: 48
SU-1: 72
A. ORYZAE: S48
A. ORYZAE: S72
A. ORYZAE: T48
A. ORYZAE: T72
9.5
7.5
4.4
2.4
1.4
.24


B
SU-1: 48
SU-1: 72
2112: 48
2112: 72
1059: 48
1059: 72
A. SOJAE: 48
A. SOJAE: 72
2115: 48
2115: 72
1273: 48
1273: 72
9.5
7.5
4.4
2.4
1.4
.24

Discussion

This study showed that *Aspergillus* species which are deficient in aflatoxin production under the conditions studied, do possess genes likely to be homologues of *nor*-1 and *ver*-1A (known to be involved in the synthesis of aflatoxin). Among the species represented were the industrial fermentation species *A. oryzae* and *A. sojae*. The aflatoxin producers (SU-1, 1059, and 1273) which also have genes homologous to *nor*-1 and *ver*-1A (as determined by Southern analyses) were confirmed to produce aflatoxins.

The production of AFB_1 (per milligram of mycelia) by these strains was greater in stationary cultures than it was in shake cultures (30 fold for SU-1). These data suggest that quantitative studies using cultures grown under shake conditions may provide an underestimate of the actual aflatoxin producting capability. Under both stationary and shake culture conditions, SU-1 produced more toxin than 1059 or 1273 at the same time point. Toxin production by 1273 was greater than 1059 in stationary cultures (48 and 72 hours), whereas the opposite was true in the shake cultures.

Northern analyses showed that a transcript pattern similar to previous reports (Trail et al, 1995) for aflatoxin producers was detected in the SU-1, 1059, and 1273 strains. First, SU-1 cultures produced more transcripts capable of hybridizing the *nor*-1 and *ver*-1A probes than did the 1059 and 1273 cultures. These results were true for stationary and shake cultures at 48 and 72 hours. The 1059 shake cultures appeared to produce more transcript than the corresponding 1273 shake cultures. This was also true for the stationary cultures even though the 1273 cultures produced more aflatoxin under

these conditions.

Finally, it was shown that none of the aflatoxin non-producers (SRRC 2112, 2115, *A. sojae*, *A. oryzae*) did not exhibit transcripts of equivalent size to those produced by SU-1. In fact, no signals were detected in the stationary cultures of the aflatoxin non-producers. A "blob", however, was detected in the 48 hour shake culture of 2112 using all of the probes listed in Table 3. It is likely that this non-specific product is not important to aflatoxin biosynthesis based on its lack of specificity. Similar sized blobs occurred for two other shake cultures (2112 and *A. sojae* at 72 hours) but they only appeared when the *ver*-1A probe was used. This suggested that there may be some irregular expression of *ver*-1A occurring in these strains at the later time point. This signal does appear to be degrading which indicates a non-stable transcript. This non-stable transcript may be degraded as the result of premature termination of transcription due to a loose association of RNA polymerase with DNA, or a mutation in a *ver*-1A like gene. Because there was no accumulation of versicolorin A (the intermediate converted in part by *ver*-1A to sterigmatocystin) nor any other intermediates in the pathway when growing this strain, it is likely that this transcript is the result of abnormal transcription, RNA processing, or RNA stability. Whether this abnormality is caused by a mutation in the promoter or coding region of the *ver*-1A gene or in a regulator is not known. An investigation (see Appendix C) of this transcript may be helpful in the identification of the factors involved in the secondary metabolism of aflatoxin.

Much has been written on the topic of secondary metabolites. Discussions about the purposes of these compounds and their origins have been at the forefront of the speculation. This study attempted to shed light on these questions as well as to investigate

aflatoxin production in suspected aflatoxin non-producers. One of two possibilities seemed likely, either these strains never had the ability to produce aflatoxins or they lost that ability. By demonstrating that these strains do have both early and late aflatoxin genes it is reasonable to say that they did produce aflatoxin at one time. So what happened to aflatoxin production? To understand what happened it is imperative to ask why any strain would produce aflatoxins.

It is generally thought that the production of a secondary metabolite facilitates growth and/or propagation of the species producing it (see *Gene* vol 115). For example, it has been speculated that antibiotic production provides strains of *Streptomyces* a defense mechanism against other microorganisms (Williams et al, 1989). Likewise, the production of sclerotia (wintering bodies) by species of *Aspergillus* provides a means of propagation for those species and possibly protection from predators - they are difficult to consume (Wicklow, 1983). Currently, the belief is that secondary metabolites were "created" as the result of gene duplications of primary metabolism pathways. This idea is based on structural similarity, amino acid identity, and organization (clustering) of genes when comparing primary and secondary pathways (Mapelstone et al, 1992). Subsequent random mutations of these genes produced various compounds that either provided a selective advantage to the organism or did not. Those organisms that did gain an advantage were better able to cope with their environment and survive. The aflatoxin biosynthetic pathway seems to fit this gene duplication theory. Based on the amino acid comparisons set out in Appendix A, the fatty acid synthase genes are closely related to their counterparts from *S. cerevisiae* involved in primary metabolism.

So at least part of the aflatoxin pathway appears to be a mutated copy of a primary

metabolism pathway. It also seems that mutations occurred by chance to produce a beneficial (to the fungi) compound. Why do many species apparently possess these genes? Once again there are two likely explanations to answer this question. The first is that the production of aflatoxins is very old. So old that at one time there was only one strain that produced it. Differentiation of that "progenitor" strain (perhaps due to dispersal to various climates) resulted in many species with the same aflatoxin production abilities but distinct morphological and growth characteristics. Another possibility is that the aflatoxin pathway was foreign to many or all species of *Aspergillus*. In this case, vertical or horizontal gene transfer provided dispersal of the aflatoxin pathway to the several *Aspergillus* spp. either from an *Aspergillus* specie (vertical) or from an organism outside the *Aspergillus* genus (horizontal). Amino acid and nucleotide analyses of genes involved in aflatoxin synthesis has revealed that genes from different species share a high degree of identity. The *afl*-R genes from *A. flavus* and *A. parasiticus*, for example, are nearly identical showing 95% identity at the nucleotide level (Payne et al, 1993). In contrast, Appendix B shows that the *pyr*G (primary metabolism gene) from *Aspergillus parasiticus* is only 78% identical to that of *A. nidulans* at the amino acid level. This indicates that the pathway has not been in the individual species for very long or that genes involved in AFB_1 synthesis are even more highly conserved than some basic cell functions (unlikely). This lends credence to the gene transfer hypothesis which could have occurred much more recently than the divergence of a progenitor strain.

One method for a horizontal gene transfer would be by a viral infection. Currently, there are few reports of viral particles found in *Aspergillus* spp. (Schmidt et al, 1986; Wood et al, 1974). Nevertheless, horizontal gene transfer should be a detectable

possibility. As has been done for *Streptomyces* (Vining, 1992; Bibb et al, 1989; Sherman et al 1989), secondary metabolite genes can be examined for codon usage, %GC content, and amino acid identity and then compared to other species from the genus *Aspergillus* and other genera. These analyses should point to the origin of the pathway whether it was completely foreign to *Aspergillus* spp. or if it spread from one species of *Aspergillus*.

Why do some strains produce aflatoxin and other strains, which apparently have at least some of the aflatoxin genes, do not? If the hypothesis that secondary metabolites provide a selective advantage is true then what happens if the pressure that produced the pathway is removed? Bennett and Goldblatt (1973) claimed that if an aflatoxin producing strain is grown in a laboratory culture and repeatedly passed to fresh medium that aflatoxin production is lost. This production, however, can be restored by growing the fungus on a plant. This may suggest that the selective advantage conveyed by aflatoxin production involves colonization. It is unclear how this could be, due to the fact that secondary metabolites are usually not produced during primary growth. Nevertheless, the most important information that can be taken from this observation (from an evolutionary standpoint) is that when the fungus is given everything it needs to grow (nutrients and conditions) in a non-competitive environment it stops making aflatoxins. This may, in fact, be what happened to some domesticated fermentation strains of *A. oryzae* and *A. sojae*. Kurtzman et al (1986) have reported that DNA reassociation studies indicate that *A. oryzae* and *A. sojae* are directly "descendant" from *A. flavus* and *A. parasiticus* respectively. Wicklow (1983) also addresses this point by noting that *A. flavus* and *A. parasiticus* strains begin to resemble *A. oryzae* and *A. sojae* during passage. (Interestingly, Wicklow also observed the loss of sclerotia production by strains that had

been passaged.) It is therefore, entirely reasonable that if the pressure that created the aflatoxin pathway was removed long enough, silent mutations to the aflatoxin pathway may have accumulated. This may have lead to the knock out of the pathway's activity.

To that end, it has been shown (Cotty, 1988; Diener et al, 1987) that the species most often found associated with crops (with the exception of peanuts) is *A. flavus*, whereas *A. parasiticus* is most often found in the soil (with the same exception). As stated earlier, not all strains of *A. flavus* produce aflatoxin, whereas all but one strain of *A. parasiticus* produce aflatoxin. It could be possible that crops such as cotton and corn place less pressure on the *A. flavus* strains that inhabit them than does the soil which *A. parasiticus* inhabits. This would explain why *A. flavus* aflatoxin non-producers are not selected against on crops. It would also explain why all *A. parasiticus* strains continue to produce aflatoxins.

APPENDICES

APPENDIX A

Appendix A - Sequence analysis of the *fas*-1A, *fas*-2A, and clone 6

Understanding how aflatoxins are produced was the most important aspect in determining what approach to take to eliminate them. Characterizing the conditions of growth and aflatoxin production led to the belief that removing aflatoxins from the food chain would not be accomplished by classical methods (farming techniques) due to the ubiquity of the producing strains. As a result it was necessary to identify the genetic makeup of the aflatoxin biosynthetic pathway. The first gene identified was the *nor*-1 gene. This gene was isolated by complementing a mutant strain (ATCC 24690, nor-) with a fragment of DNA containing a wild type copy of the gene (Chang et al, 1992). The next gene to be identified was *ver*-1A (Skory et al, 1992). Shortly after the isolation of these genes a 35-kb fragment of genomic DNA, the cosmid NorA (Figure 10), capable of complementing both of these mutants was defined (Trail et al, 1995). Restriction analyses of the NorA cosmid yielded 11 subfragments (clones). Using parts of each of these clones as probes, northern hybridization analysis was carried out to determine the number and size of the transcripts being produced by this cosmid. In addition, these analyses were carried out at various time points to determine the timing of expression. To associate the timing of expression of each of the transcripts with production of aflatoxin, ELISA analysis was performed as described in the materials and methods (Trail et al, 1995).

In a collaboration with researchers at the SRRC ARS USDA, 10 of the 11 clones from the cosmid NorA were sent to DNA Technologies (Gaithersburg, MD) to be sequenced. Analysis of this sequence has revealed the presence of a putative fatty acid

synthase (*fas*-1A) encoded by clones two, one, and eight (Mahanti et al, 1996). Comparisons using TFASTA, GAP, and PILEUP from the Wisconsin Genetics Computer Group (WGCG) software package turned up high identity, at the amino acid level, with the *Saccharomyces cerevisiae fas*1 gene (Schweizer et al, 1986). Identification of each of the functional domains (acetyl transferase, enoyl reductase [Mahanti et al, 1996], dehydratase, and malonyl/palmityl transferase [Mahanti et al, 1996]) found in the *S. cerevisiae* gene was accomplished in the *fas*-1A (Figures 11, 12).

The fatty acid synthase in *S. cerevisiae* is actually composed of two subunits, the beta subunit (*fas*1, mentioned above) and the alpha subunit - the *fas*2 (Mohamed et al, 1988). The *fas*2 encodes the final three functional domains needed for fatty acid synthesis (acyl carrier protein, β-ketoacyl reductase, and β-ketoacyl synthase). Again, sequence analysis of a region (clone2 and the 11th clone "4.6" [sequenced by Trail]) of the cosmid NorA showed high identity to the published *fas*2 at the amino acid level (Figures 13, 14, 15). The order of the domains in the *S. cerevisiae* genes is identical to that of the *A. parasiticus* genes. Also, the spacing between the domains and the active site amino acids are virtually identical between the two species. The comparisons also included an alignment with the *Streptomyces antibioticus* polyketide synthase which has been implicated in the synthesis of oleandomycin (Swan et al, 1994). The nucleotide sequence of the putative alpha subunit (*fas*-2A) is given in Figure 16.

Being that regulators typically act at promoter regions it is interesting to find that *fas*-1A and *fas*-2A appear to be transcribed divergently (Figure 10). Furthermore, based on sequence analysis, the domains from each of these genes that are closest to each other (the acetyl transferase in *fas*-1A and the acyl carrier protein in *fas*-2A) may have

translational start codons within 200 base pairs of each other. This leaves a rather small area for regulator binding and may suggest that the promoter regions of these genes overlap or are shared.

Finally, sequence analysis of the sixth clone from the cosmid NorA has revealed a match (Figure 17) with 188 amino acids (including a heme binding site residue, cystine) of cytochrome P450 from rabbit (27% identity, 49% similarity) and rat (26% identity, 46% similarity). This gene is likely one of the many oxidases used in the production of aflatoxins (Bhatnagar et al, 1992). Keller et al (1995) demonstrated that the *ver*-1A homologue (*stc*U) in *A. nidulans* requires a functional *stc*S to convert versicolorinA to sterigmatocystin. *stc*S, located approximately 2.0-kb from *stc*U and transcribed off the same DNA strand, is proposed to be a cytochrome P450. This situation is similar to that seen between *ver*-1A and the clone 6 cytochrome P450. It is very likely that as *stc*U and *ver*-1A are homologues so too are *stc*S and the gene encoded by clone 6. Once again an interesting note bares mention. Figure 17 shows that there is a match with cytochrome P450 from rabbit (Johnson et al, 1990) and rat (Chen and Hardwick, 1993) but the comparison with the fungus *Streptomyces carbophilus* (Wantanabe et al, 1995) does not show a significant match (17% identity, 35% similarity). This raises questions about the function of this gene. Does it play a role in aflatoxin production? If so, the presence of this gene may suggest that the biosynthetic pathway did not evolve from another pathway in the organism such as a PKS. It would support, however, the theory of gene transfer. The nucleotide sequence of the cytochrome P450 like sequence of clone 6 is presented in Figure 18.

Figure 10. Map of the Cosmid NorA.

The Cosmid Nor A

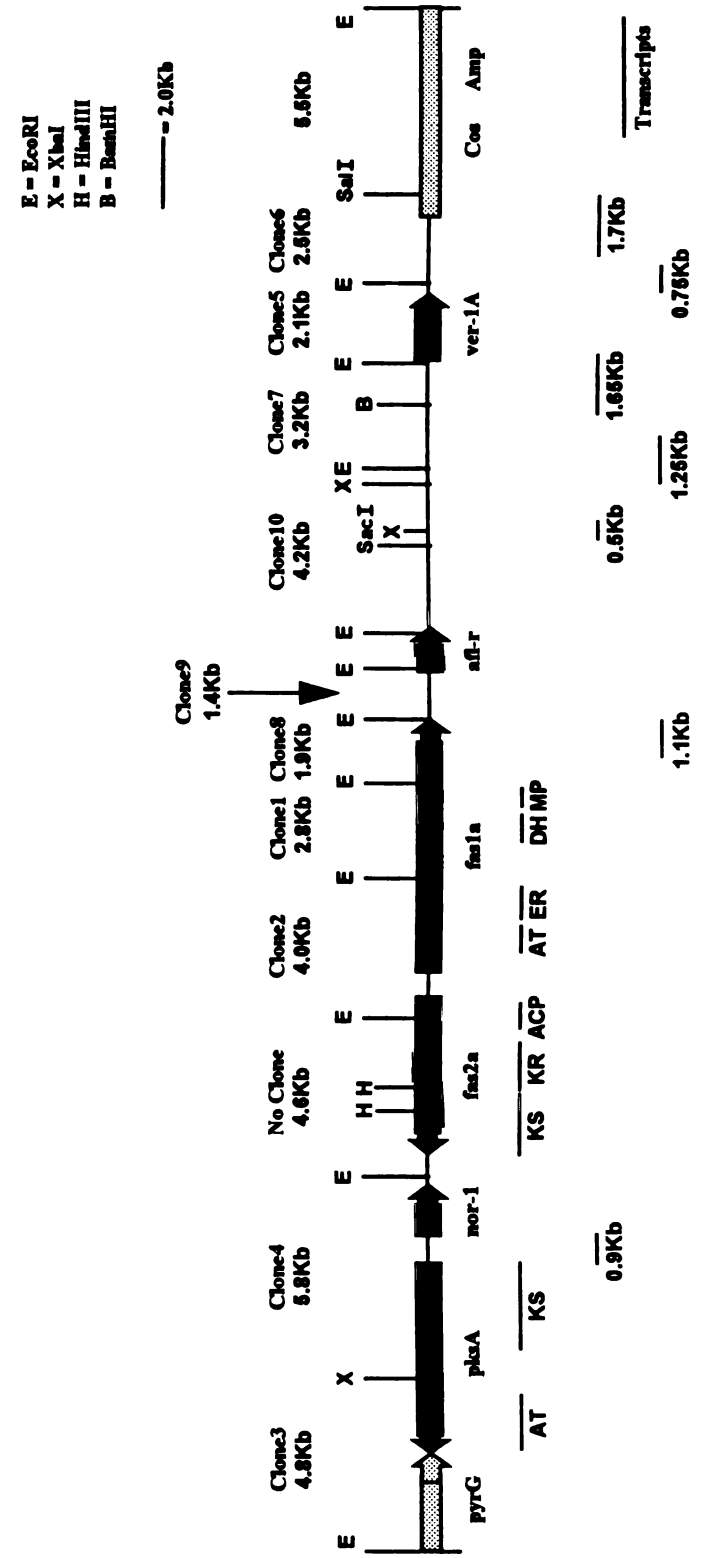


Figure 11. Amino acid comparison of the suspected acetyl transferase domain of *fas*-1A of *A. parasiticus* (AP) with *S. cerevisiae* (SC, a FAS) and *S. antibioticus* (SA, a PKS). The *A. parasiticus* acetyl transferase domain was 27% identical and 42% similar to the same domain in *S. cerevisiae* over 279 amino acids, but only 16% identical and 42% similar to the acetyl transferase of *S. antibioticus* over the same stretch. The active site residue is marked by a down arrow. Regions of the SA were removed to optimize the alignment. Those regions are marked by "> <" and correspond to the following stretches of amino acid sequence in the published SA: 729 - 738, 811 - 829, 867 - 882, and 2333 - 2355.

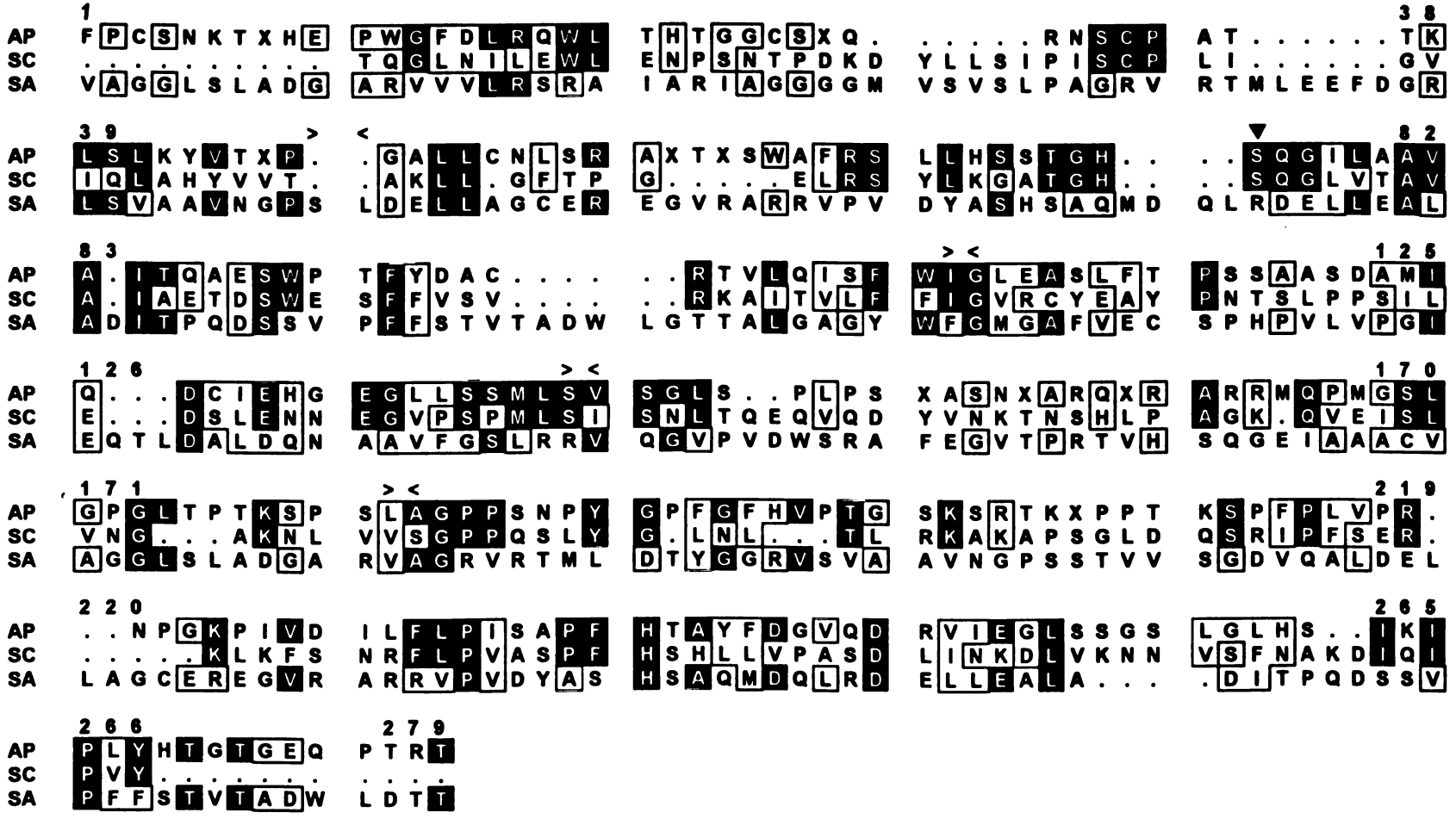


Figure 11.

Figure 12. Amino acid comparison of the suspected dehydratase domain from the *fas*-1A of *A. parasiticus* (AP) with *S. cerevisiae* (SC, a FAS) These two amino acid sequences lined up over 196 amino acids resulting in 27% identity and 43% similarity. Two stretches of the SC were removed to optimize the alignment. Those omissions are marked by "> <" and correspond to the following regions in the published SC sequence: 1456 - 1480 and 1425 - 1435.

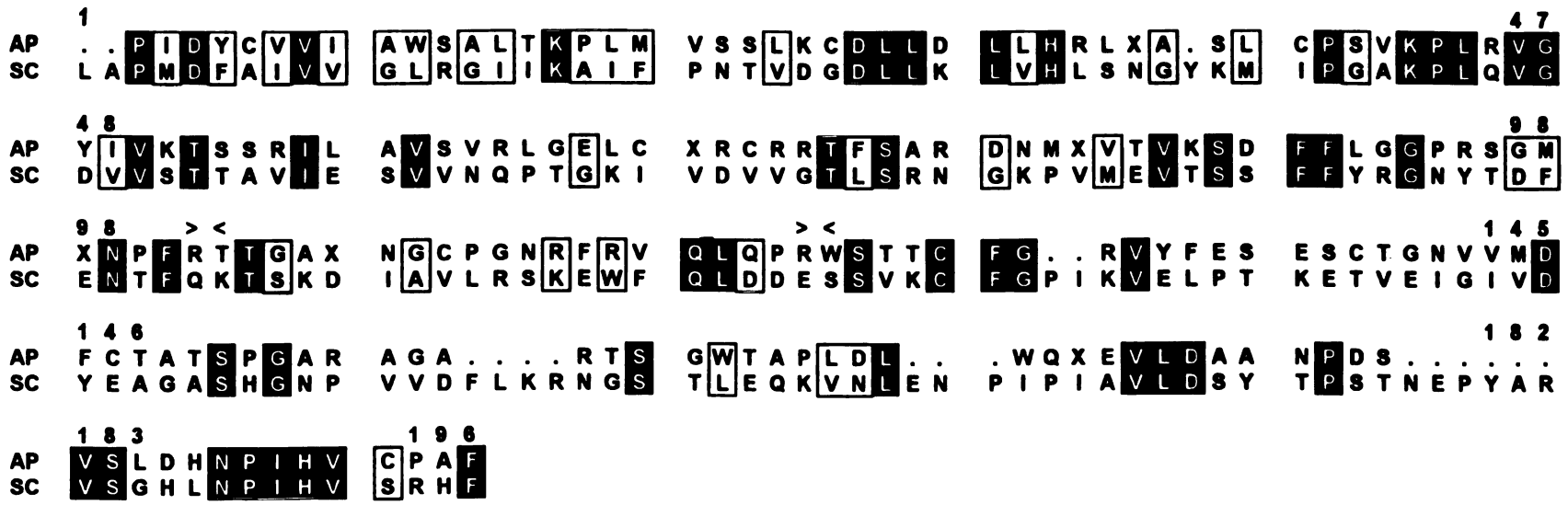

1
AP . . P I D Y C V V I A W S A L T K P L M V S S L K C D L L D L L H R L X A . S L C P S V K P L R V G
SC L A P M D F A I V V G L R G I I K A I F P N T V D G D L L K L V H L S N G Y K M I P G A K P L Q V G
47
48
98
AP Y I V K T S S R I L A V S V R L G E L C X R C R R T F S A R D N M X V T V K S D F F L G G P R S G M
SC D V V S T T A V I E S V V N Q P T G K I V D V V G T L S R N G K P V M E V T S S F F Y R G N Y T D F
98
><
145
AP X N P F R T T G A X N G C P G N R F R V Q L Q P R W S T T C F G . . R V Y F E S E S C T G N V V M D
SC E N T F Q K T S K D I A V L R S K E W F Q L D D E S S V K C F G P I K V E L P T K E T V E I G I V D
146
182
AP F C T A T S P G A R A G A . . . . R T S G W T A P L D L . . . W Q X E V L D A A N P D S . . . . . .
SC Y E A G A S H G N P V V D F L K R N G S T L E Q K V N L E N P I P I A V L D S Y T P S T N E P Y A R
183
196
AP V S L D H N P I H V C P A F
SC V S G H L N P I H V S R H F

Figure 12.

Figure 13. Amino acid comparison of the suspected acyl carrier protein domain from the *fas*-2A of *A. parasiticus* (AP) with *S. cerevisiae fas*2 (SC, a FAS) and *S. antibioticus* (SA, a PKS). The *A. parasiticus* acyl carrier protein domain was 34% identical and 56% similar to the same domain in *S. cerevisiae* over 271 amino acids, but only 13% identical and 27% similar to the acyl carrier protein of *S. antibioticus* over the same stretch. The active site is marked by a down arrow. Two stretches of the SC were removed to optimize the alignment. Those omissions are marked by "> <" and correspond to the following regions in the published SC sequence: 336 - 344 and 359 - 370.

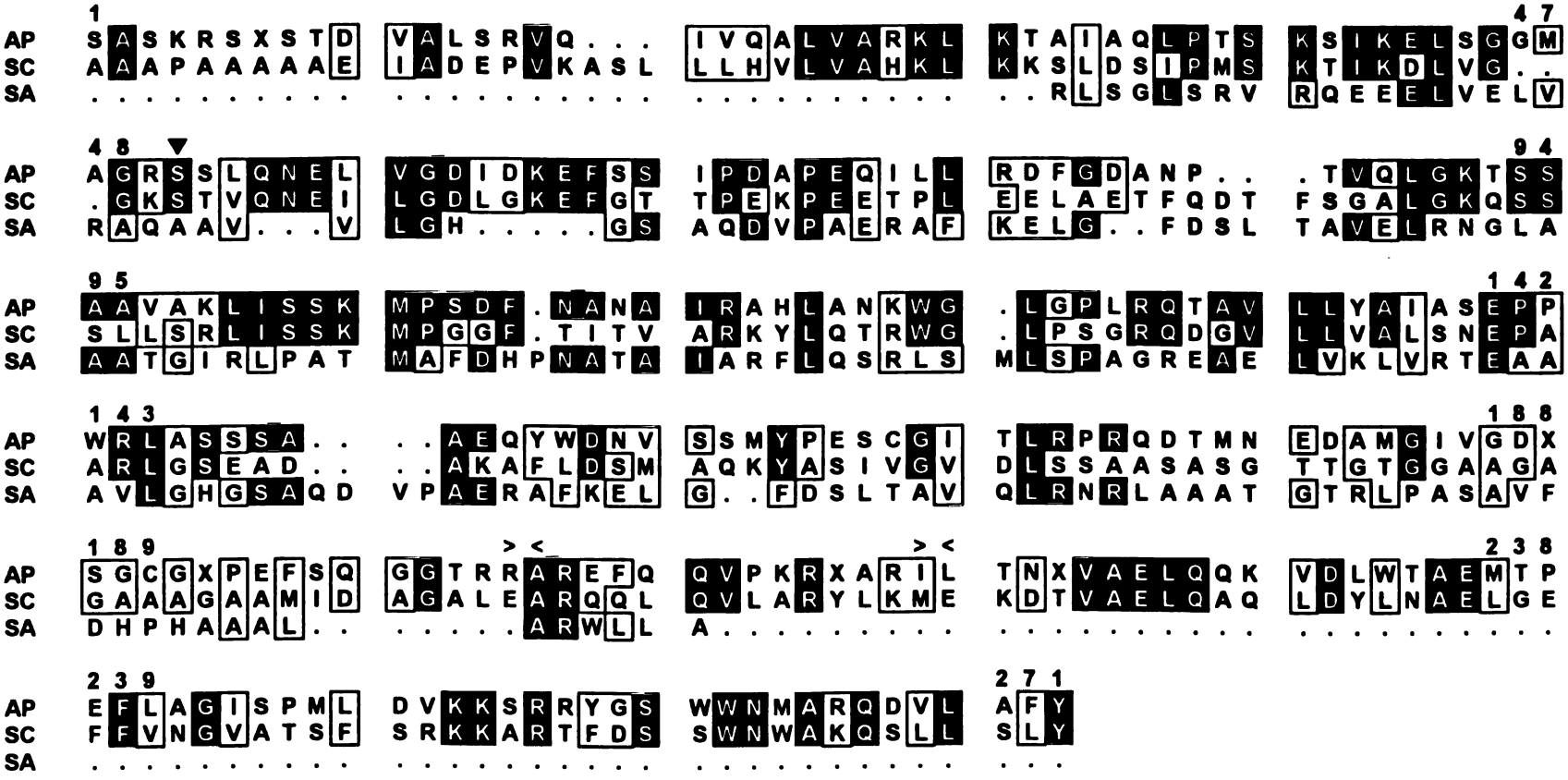


Figure 13.

Figure 14. Amino acid comparison of the suspected β-ketoacyl reductase domain from the *fas*-2A of *A. parasiticus* (AP) with *S. cerevisiae* (SC, a FAS) and *S. antibioticus* (SA, a PKS). The *A. parasiticus* β-ketoacyl reductase domain was 42% identical and 62% similar to the same domain in *S. cerevisiae* over 479 amino acids, but only 14% identical and 34% similar to the β-ketoacyl reductase of *S. antibioticus* over the same stretch. The active site is marked by a down arrow. One segment from the SC and SA was removed to optimize the alignment. Those omissions are marked by "> <" and correspond to the following regions in the published sequences: SC, 946 - 1012 and SA, 915 - 2937.

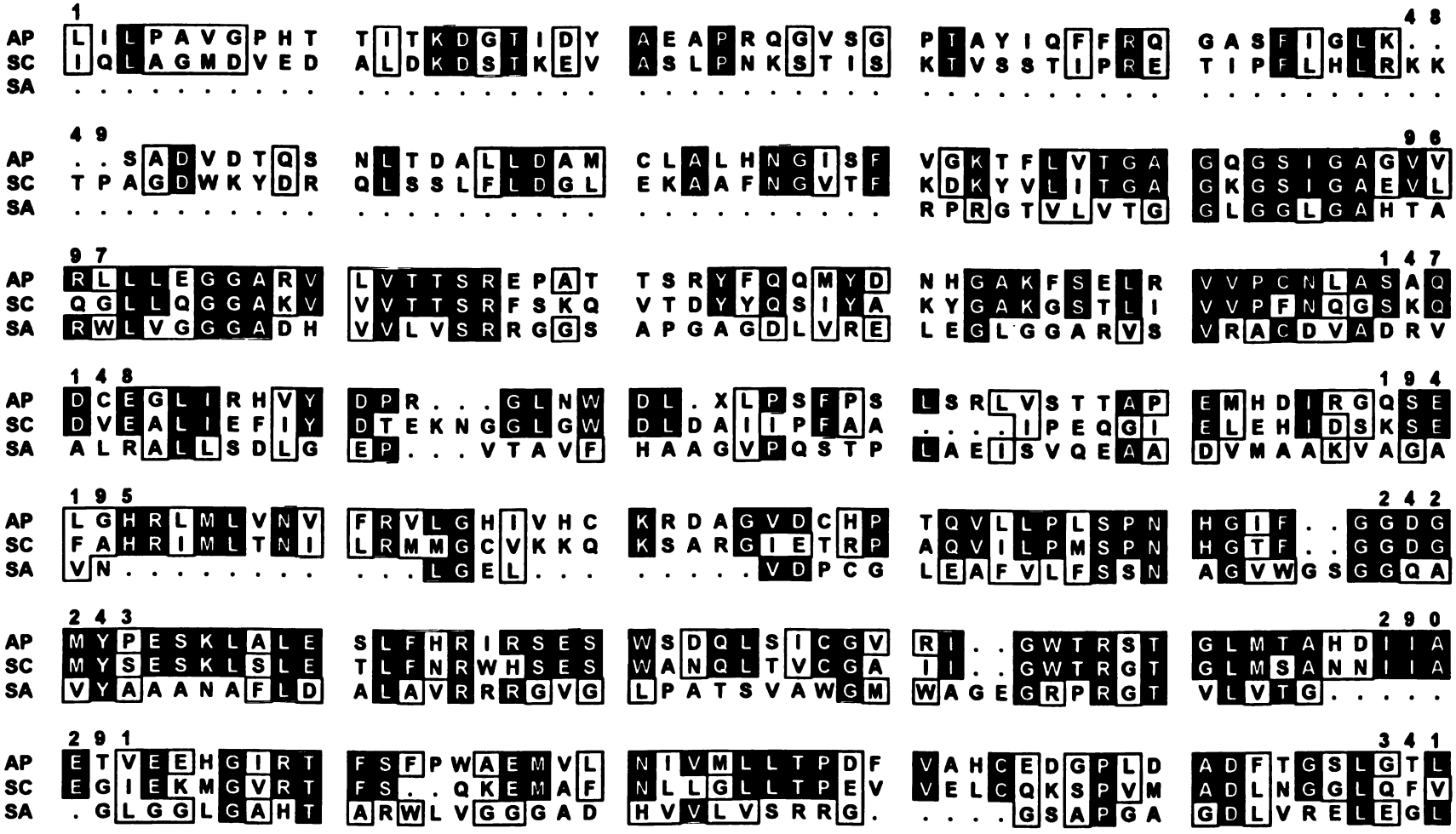


Figure 14.

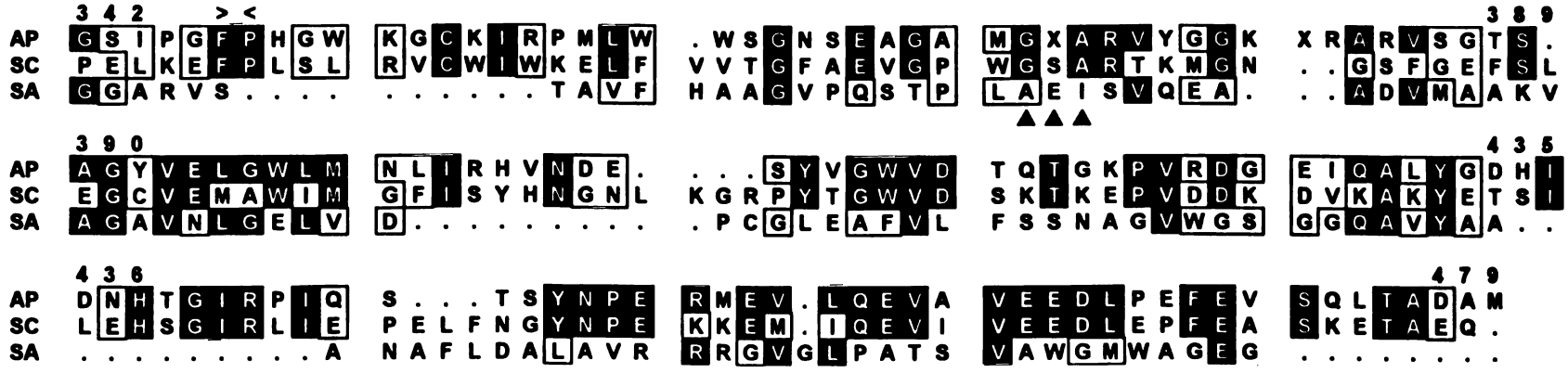


Figure 14.

Figure 15. Amino acid comparison of the suspected β-ketoacyl synthase domain from the *fas*-2A of *A. parasiticus* (AP) with *S. cerevisiae* (SC, a FAS) and *S. antibioticus* (SA, a PKS). The *A. parasiticus* β-ketoacyl synthase domain was 41% identical and 61% similar to the same domain in *S. cerevisiae* over 631 amino acids, but only 19% identical and 37% similar to the β-ketoacyl synthase of *S. antibioticus* over the same stretch. The active site is marked by a down arrow. One segment from the SC and several from the SA were removed to optimize the alignment. Those omissions are marked by "> <" and correspond to the following regions in the published sequences: SA, 52 - 60, 101 - 108, 318 - 334, 341 - 434; SC, 1435 - 1520; SA, 461 - 468, 1709 - 1715, 1807 - 1812, 1843 - 1850, 1954 - 1960, 1996 - 2007, 2049 - 2067.

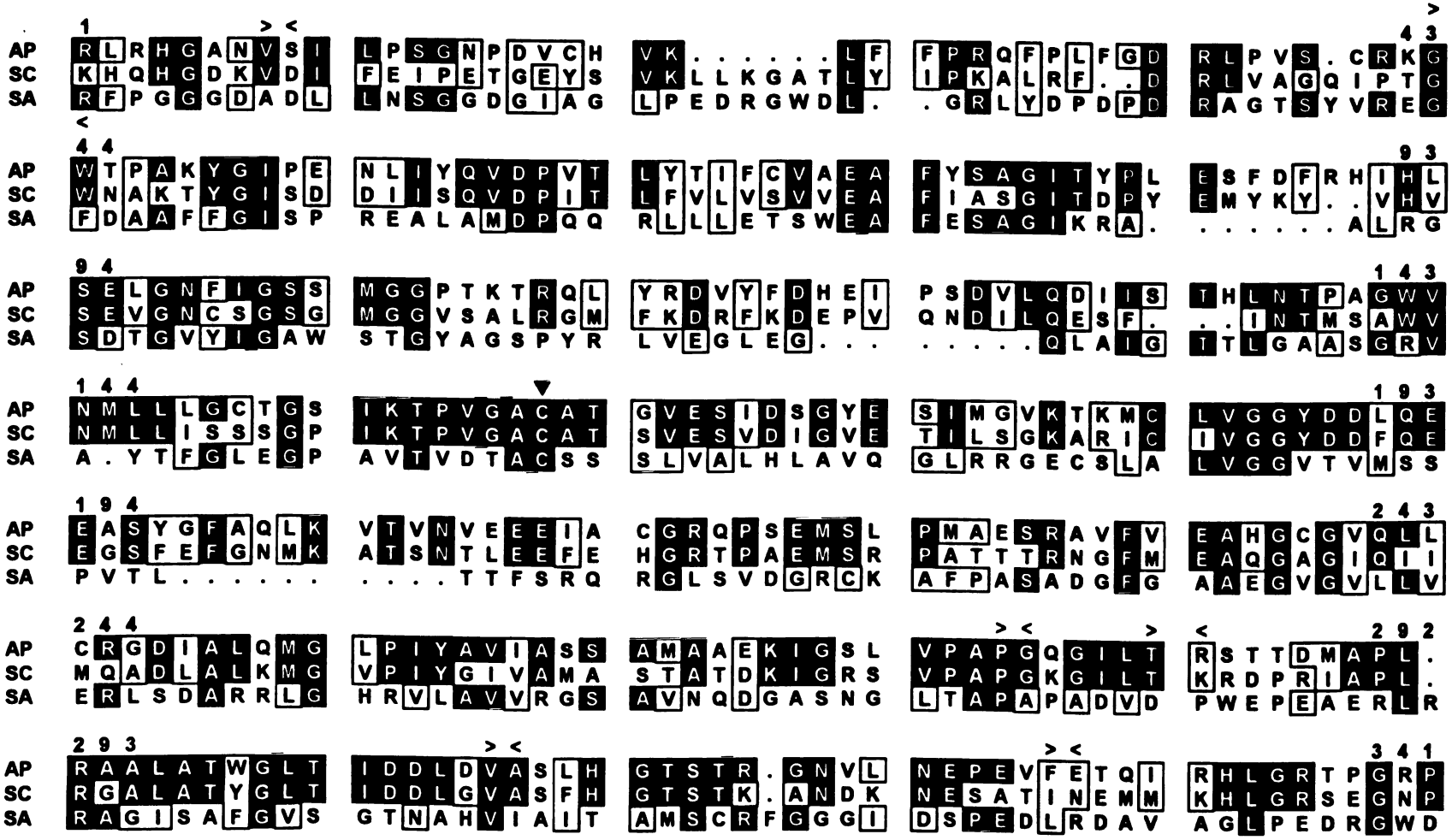


Figure 15.

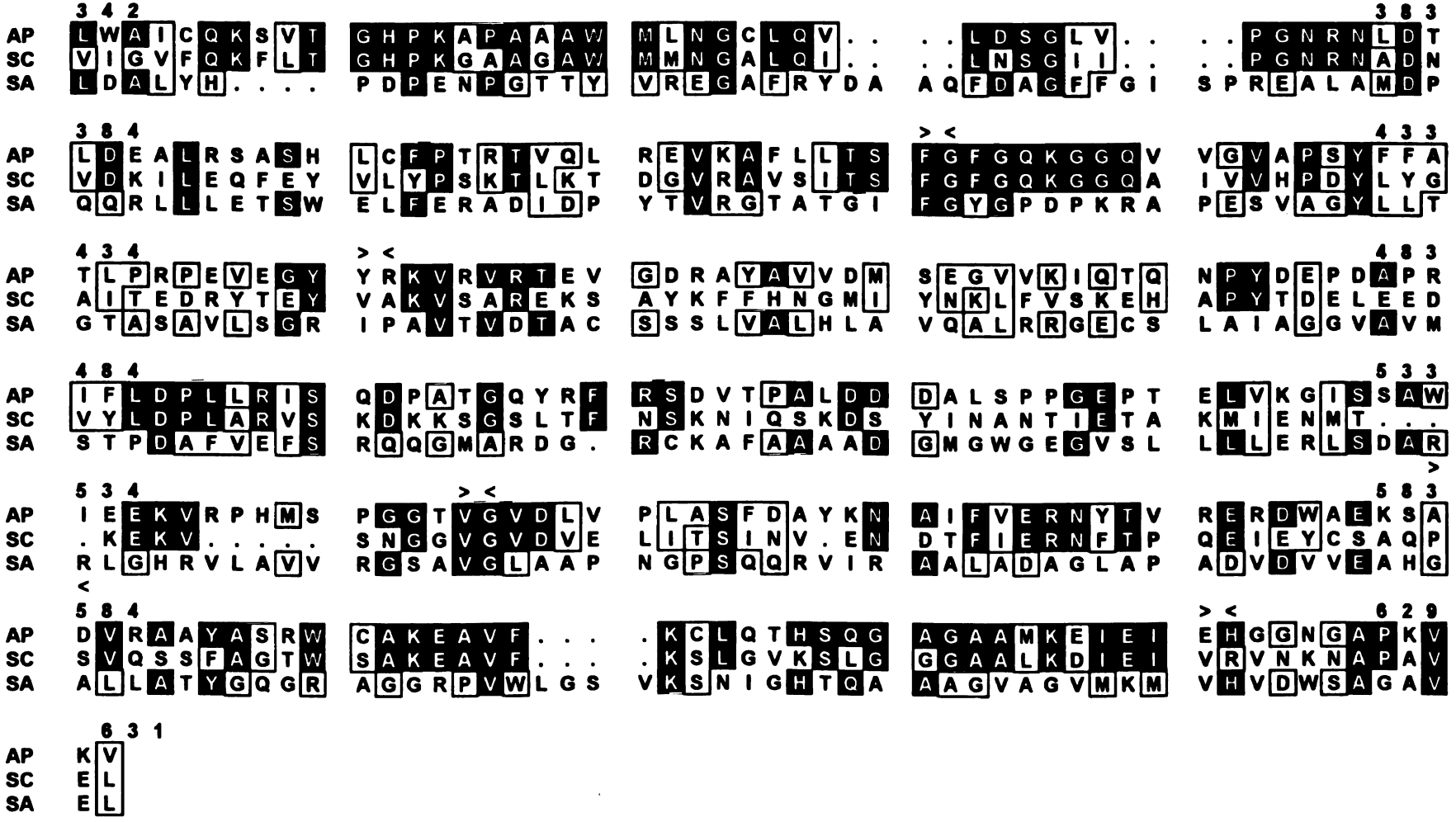
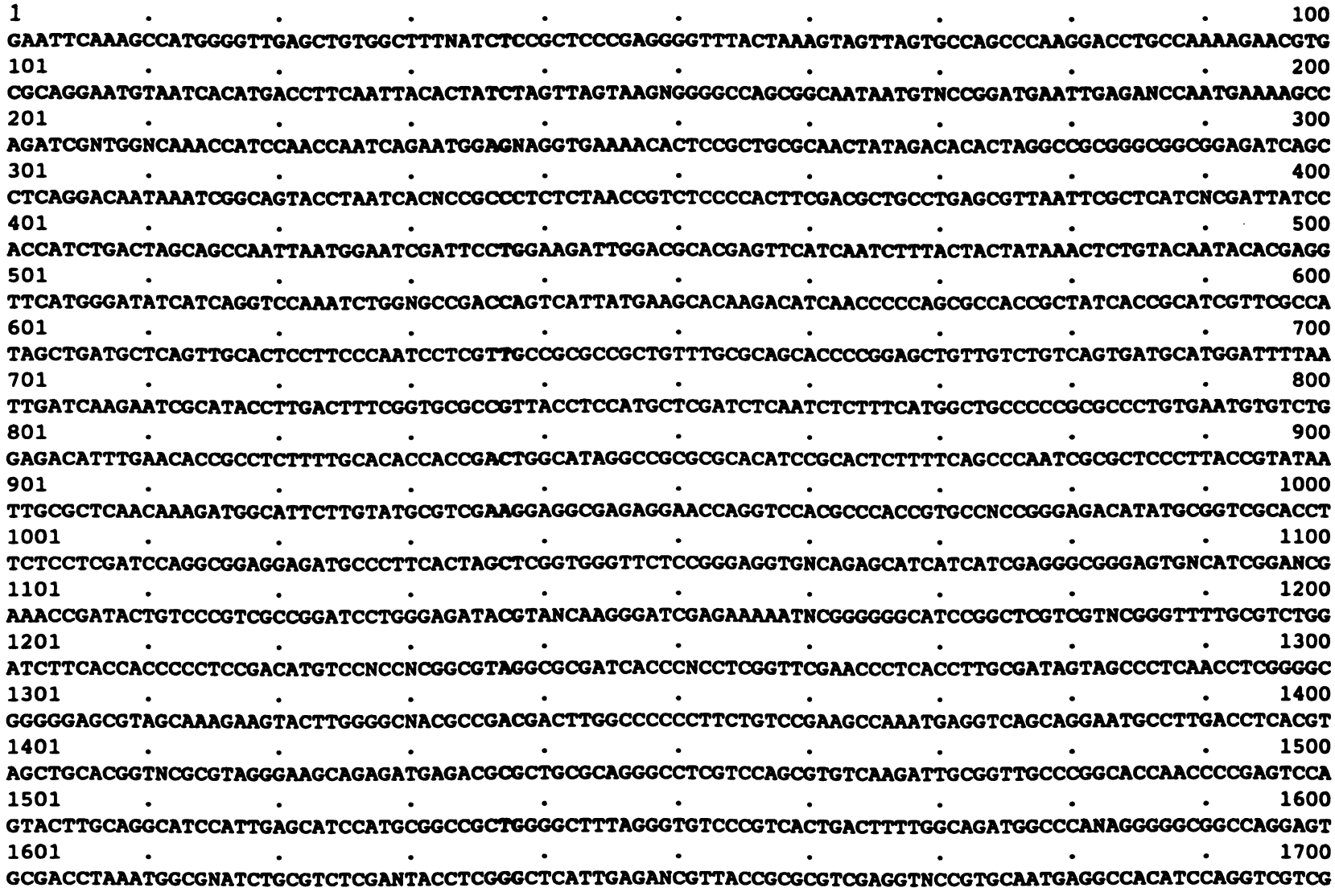


Figure 15.

Figure 16. Nucleotide sequence of the *fas*-2A of *A. parasiticus*.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301 400
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401 500
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701 800
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901 1000
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1001 1100
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1301 1400
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1501 1600
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1601 1700
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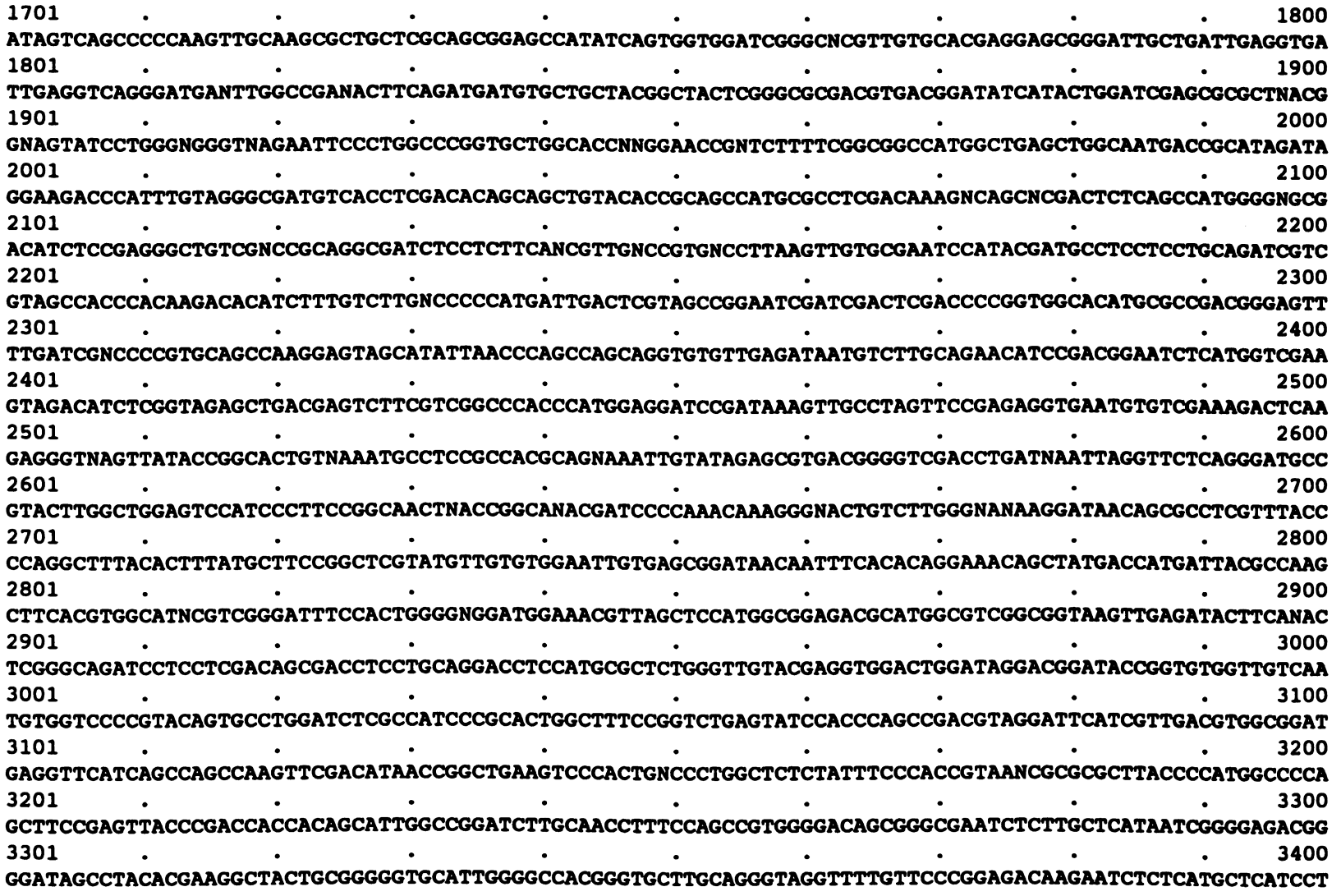
Figure 16.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2001 2100
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2101 2200
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2201 2300
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2501 2600
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2601 2700
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2701 2800
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2901 3000
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3201 3300
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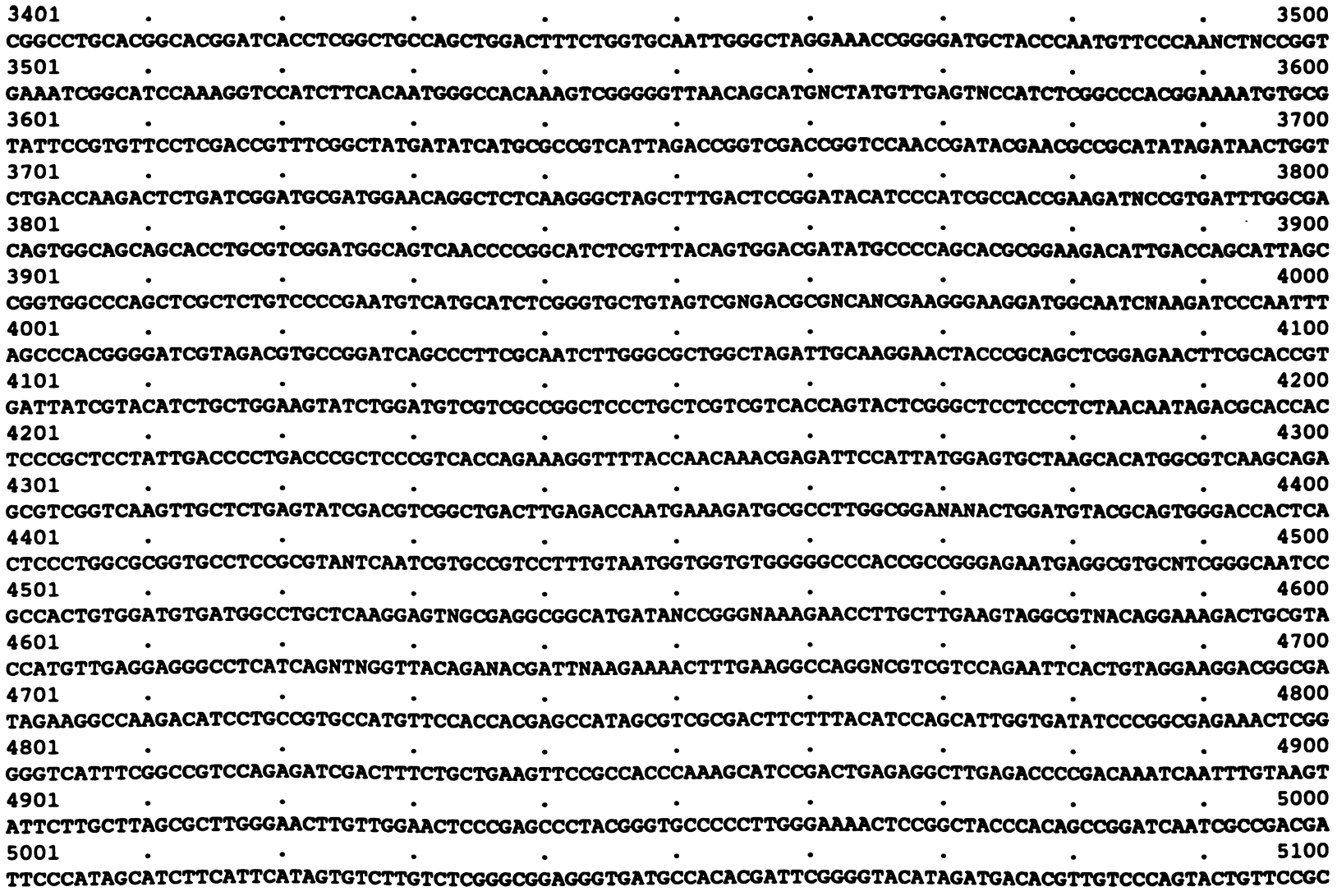
Figure 16.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3801 3900
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3901 4000
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4001 4100
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4201 4300
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4301 4400
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4501 4600
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4601 4700
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4701 4800
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4801 4900
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4901 5000
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5001 5100
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Figure 16.

```
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5401      .         .         .         .         .         .         .         .     5500
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5601      .         .         .         .         .         .         .         .     5700
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5701      .         .         .         .         .         .         .         .     5800
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5801      .         .         .         .         .         .         .         .     5900
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5901      .         .         .         .         .         .         .         .     6000
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6001      .         .         .         .         .         .         .         .     6100
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6101      .         .         .         .         .         .       6174
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```

Figure 16.

Figure 17. Amino acid comparison of a suspected cytochrome P450 domain from clone 6 of *A. parasiticus* (ASPER) with rabbit (RABBIT), rat (RAT), and *Streptomyces carbophilus* (STREP)(see text).

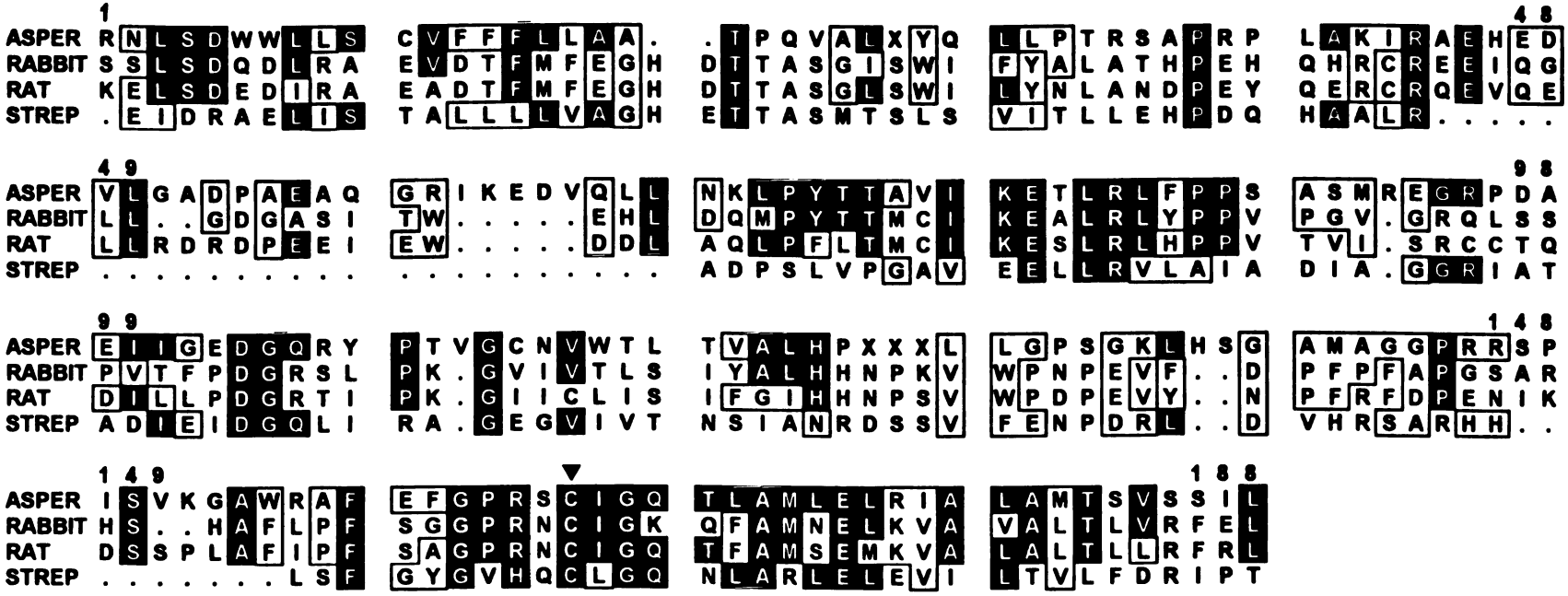

Figure 17.

Figure 18. Nucleotide sequence of the cytochrome P450 like region of clone 6 from the Cosmid NorA. The total clone 6 sequence is 2489 nucleotides but less than 2000 bases of this sequence is from the Cosmid NorA, the rest is vector sequence (pBZ5).

```
1                                                                   100
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201                                                                 300
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301                                                                 400
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401                                                                 500
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501                                                                 600
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601                                                                 700
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701                                                                 800
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801                                                                 900
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901                                                                 1000
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1001                                                                1100
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1201                                                                1300
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1301                                                                1400
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1401                                                                1500
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1501                                                                1600
TCCCTCCATCAGCATCGATGCGGGAGGGCCGGCCTGATGCTGAAATCATTGGAGAAGACGGTCAACGATATCCCACGGTGGGGTGCAACGTCTGGACGCT
1601                                                                1700
CACGGTGGCTCTCCACCCATAATAGTGACTACTGGGACCAAGTGGAAAACTTCATTCCGGAGCGATGGCTGGTGGGCCCCGAAGATCCCCTATATCGGTT
```

Figure 18.

```
1701      .         .         .         .         .         .         .         .      1800
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1801      .         .         .         .         .         .         .         .      1900
GTTCGATATTACGCCAGCGTTACGATGAGTGGGACAGCATTCACCCAGCTACTACTGCCAAAGAGGTTAATGGGCATCGTGCTACCAGCGGAGAGAGGCG
```

Figure 18.

In the following manuscript I was responsible for analyzing the amount of aflatoxin produced by the strains using ELISA. I also generated the resulting graphs. Finally, I performed the sequence analysis of the suspected PKS using the Wisconsin Genetics Computer Group software; I created the comparison figure using Quattro Pro for Windows version 5.0.

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0099-2240/95/$04.00+0

Physical and Transcriptional Map of an Aflatoxin Gene Cluster in *Aspergillus parasiticus* and Functional Disruption of a Gene Involved Early in the Aflatoxin Pathway

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Received 6 January 1995/Accepted 20 April 1995

Two genes involved in aflatoxin B_1 (AFB1) biosynthesis in *Aspergillus parasiticus*, *nor-1* and *ver-1*, were localized to a 35-kb region on one *A. parasiticus* chromosome and to the genomic DNA fragment carried on a single cosmid, NorA. A physical and transcriptional map of the 35-kb genomic DNA insert in cosmid NorA was prepared to help determine whether other genes located in the *nor-1–ver-1* region were involved in aflatoxin synthesis. Northern (RNA) analysis performed on RNA isolated from *A. parasiticus* SU1 grown in aflatoxin-inducing medium localized 14 RNA transcripts encoded by this region. Eight of these transcripts, previously unidentified, showed a pattern of accumulation similar to that of *nor-1* and *ver-1*, suggesting possible involvement in AFB1 synthesis. To directly test this hypothesis, gene-1, encoding one of the eight transcripts, was disrupted in *A. parasiticus* CS10, which accumulates the aflatoxin precursor versicolorin A, by insertion of plasmid pAPNVES4. Thin-layer chromatography revealed that gene-1 disruptant clones no longer accumulated versicolorin A. Southern hybridization analysis of these clones indicated that gene-1 had been disrupted by insertion of the disruption vector. These data confirmed that gene-1 is directly involved in AFB1 synthesis. The predicted amino acid sequence of two regions of gene-1 showed a high degree of identity and similarity with the β-ketoacyl-synthase and acyltransferase functional domains of polyketide synthases, consistent with a proposed role for gene-1 in polyketide backbone synthesis.

Aflatoxins are potent teratogenic, mutagenic, and carcinogenic secondary metabolites synthesized by certain strains of *Aspergillus parasiticus* and *A. flavus* (25). Under the proper environmental conditions, these ubiquitous fungi can produce aflatoxin upon infection of many agricultural crops, including peanuts, corn, cottonseed, and tree nuts (20). Because of the difficulty in effectively controlling aflatoxin contamination of food and feed by traditional agricultural practices, recent research efforts have focused on developing an understanding of the molecular biology of the aflatoxin biosynthetic pathway. This knowledge may lead to novel methods for control of this economically and agriculturally important problem.

Aflatoxins are polyketide-derived secondary metabolites. The carbon backbone of aflatoxin B_1 (AFB1) is synthesized from acetate and malonate in a process analogous to fatty acid synthesis (9, 24, 53). A generally accepted pathway for the synthesis of AFB1 has been proposed (reviewed in references 9 and 10). The first stable intermediate identified in the pathway is the decaketide norsolorinic acid (NA), an anthraquinone, which is converted to averufin (AVF) by a multistep series of reactions involving up to three alternative pathways (9, 56). AVF is then converted to versiconal hemiacetal acetate, versiconal, versicolorin B, versicolorin A (VA), demethylsterigmatocystin, sterigmatocystin (ST), *O*-methylsterigmatocystin, and finally, to AFB1. As many as 17 different enzyme activities are proposed to be involved in aflatoxin synthesis (9, 24). Several of these enzymes have been purified to homogeneity (1, 8, 11, 16, 28, 34, 40, 53).

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Aflatoxin-blocked mutants (4, 32) and purified enzymes have been used to clone several genes involved in the aflatoxin biosynthetic pathway, including *nor-1* (15), encoding an activity which converts NA to averantin; *ver-1* (48), encoding an activity associated with the conversion of VA to ST; *uvm8* (36), encoding a putative fatty acid synthase involved in polyketide backbone synthesis; *omt-1*, encoding a methyltransferase which converts ST to *O*-methyl-ST (57); and *aflR* (13, 45), apparently involved in the regulation of pathway gene expression. The recombinational inactivation (gene disruption) of *nor-1* (54), *ver-1* (33), *uvm8* (36), and *pksA* (14) in *A. parasiticus* and *verA* (29) in *A. nidulans* (which synthesizes ST) firmly established the functional role of these genes in the AFB1 (or ST) biosynthetic pathway.

Parasexual analyses of eight aflatoxin-blocked mutants (including an NA-accumulating strain) in *A. flavus* suggested that all loci were genetically linked on linkage group VII (44). Attempts to demonstrate linkage of *nor-1* and *ver-1* genes in *A. parasiticus* by parasexual analyses, however, gave conflicting results (5, 12; reviewed in reference 7). The molecular genetic analysis presented in the current study clearly demonstrates the clustering (linkage) of *nor-1*, *uvm8*, and *ver-1* within a 35-kb region on one chromosome in *A. parasiticus* SU1. In addition, restriction endonuclease analysis and transcript mapping of this 35-kb region localized eight other transcripts that are expressed in a pattern similar to that of *nor-1*, *ver-1*, and *uvm8*, suggesting that the genes encoding them are also involved in aflatoxin production. To test this hypothesis, disruption of gene-1 (tentatively named because of its position at the far left end of the cluster) encoding a 7-kb transcript within the gene cluster (37) was accomplished in this study. Genetic and biochemical analyses of disruptant clones and nucleotide sequence analysis of extensive regions within gene-1 suggest that

it encodes a polyketide synthase involved in AFB1 biosynthesis.

MATERIALS AND METHODS

Strains and culture conditions. *Escherichia coli* DH5α F' '[F'*lendA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1 recA1 gyrA* (Nal') *relA1*Δ(*lacZYA-argF*)$_{u169}$ (*m80lacZ*Δ M15)] was used for propagating plasmid DNA. *A. parasiticus* NRRL 5862 (SU1), a wild-type aflatoxin-producing strain, was used for preparation of RNA for transcript mapping. *A. parasiticus* CS10 (*ver-1 wh-1 pyrG* [48]), derived from *A. parasiticus* ATCC 36537 (*ver-1 wh-1* [4]) was used as the host strain for the disruption of gene-1. Strains CS10 and ATCC 36537 are unable to convert VA to ST as a result of a *ver-1* mutation, and neither produces detectable levels of AFB1 in liquid or on solid growth media. The following strains of *A. parasiticus* were used to analyze sclerotium development: AFB1-producing strain SU1, AVF-accumulating strain ATCC 24551 (23), NA-accumulating strain ATCC 24690 (32), and VA-accumulating strain ATCC 36537.

Fungal strains were maintained as frozen spore stocks (approximately 10^8 spores per ml) in 20% glycerol at −80°C. Coconut agar medium (CAM [2]), an aflatoxin-inducing medium, was used for rapid screening of fungal strains for accumulation of AFB1 and VA by visualization of blue and yellow fluorescence, respectively, under long-wave UV light. YES broth (2% yeast extract, 20% sucrose; pH 5.5), a rich aflatoxin-inducing medium, was used to grow mycelia for DNA and RNA preparations and for thin-layer chromatography (TLC) assays. Reddy's medium, a chemically defined aflatoxin-inducing medium (47), also was used to grow mycelia for RNA preparations.

Isolation and analysis of RNA and DNA. Fungal cultures for DNA and RNA preparations were grown in YES (DNA and RNA) or Reddy's medium (RNA) at 29°C with shaking (175 rpm) and harvested at the times indicated in the figure legends (48 h for DNA). DNA was purified from *A. parasiticus* by a published modification (50) of a phenol-chloroform protocol developed for mammalian DNA (3). Total RNA was purified from aflatoxin-induced cultures of *A. parasiticus* SU1 (10^5 spores per ml), using a hot-phenol protocol previously described (39). Restriction endonucleases utilized in analysis of DNA were purchased from Boehringer Mannheim Biochemicals or New England BioLabs and were used according to the manufacturer's instructions. Northern (RNA) and Southern hybridization analyses were performed using published procedures (38), with a modified hybridization buffer and conditions recommended by Stratagene Cloning Systems, La Jolla, Calif. ^{32}P-radiolabelled DNA probes were prepared with a Random Primed DNA Labelling Kit from Boehringer Mannheim Biochemicals. After the final wash, nylon or nitrocellulose membranes were placed on X-ray film (Kodak-XAR5) at −81°C. DNA probes utilized in transcript mapping (Northern analyses) and Southern hybridization analyses were generated from DNA restriction fragments derived from cosmid NorA or cosmid subclones as shown in Fig. 1. The gene probes used as controls in transcript mapping consisted of a 4.3-kb *Eco*RI-*Sac*I DNA restriction fragment isolated from cosmid NorA containing the *pyrG* gene from *A. parasiticus* (48) and a 1.1-kb *Acc*I-*Sac*I fragment containing part of the coding region of the gene encoding β-tubulin in *A. parasiticus* (55a).

Plasmid and cosmid construction and purification. Cosmids NorA, NorB, Ver2, Ver3, and Ver4 were isolated by in situ colony hybridization (3) of a cosmid library containing *A. parasiticus* SU1 genomic DNA cloned into the cosmid vector pBZ5 (48). ^{32}P-labelled DNA restriction fragments containing the *nor-1* or *ver-1* genes (*nor-1*, 1.5-kb *Bgl*II-*Cla*I restriction fragment; *ver-1*, 0.6-kb *Ava*I-*Bam*HI restriction fragment [49]) were used as probes to screen the library.

Plasmid pAPNVES43 was constructed to disrupt gene-1 by single-crossover insertion into the homologous region of the chromosome (see Fig. 4). A 4.3-kb *Eco*RI-*Sac*I *A. parasiticus* genomic DNA restriction fragment subcloned from cosmid NorA, encompassing the entire *pyrG* coding region (part of the original pBZ5 vector) plus a 1.6-kb region within the coding region of gene-1, was subcloned into pUC19 cut with *Eco*RI and *Sac*I.

Plasmid minipreparations were performed by the boiling method (38); large-scale plasmid preparations were performed according to the alkaline lysis procedure of Maniatis et al. (38).

Restriction endonuclease analysis, transcript mapping, and physical linkage analysis of cosmid NorA. An *Eco*RI restriction endonuclease digest of cosmid NorA was prepared. The resulting fragments were subcloned into pUC19 or pBluescript SKII(−) except for a 10.6-kb *Eco*RI fragment containing the *nor-1* gene and a 4.6-kb *Eco*RI fragment immediately adjacent to the 10.6-kb fragment. The 10.6-kb *Eco*RI fragment was cut into two fragments with *Sac*I, and each resulting fragment (4.3 and 6.3 kb; clones 3 and 4, respectively) was subcloned into pBluescript SKII(−) cut with *Eco*RI and *Sac*I. The 4.6-kb *Eco*RI fragment was subcloned into pBluescriptSKII(−) as three fragments: two 1.8-kb *Eco*RI-*Hin*dIII fragments flanking one 0.6-kb *Hin*dIII fragment (see Fig. 2). From these subclones, a restriction map of the entire cosmid was prepared by mapping each subclone with *Kpn*I, *Apa*I, *Sma*I, *Sac*I, and *Xba*I (Fig. 1). The genes *nor-1*, *ver-1*, and *uvm8* were localized onto subclones by Southern hybridization analysis in conjunction with the restriction endonuclease analysis. The position of *aflR* was provided as part of a separate study (58).

Measurement of aflatoxin synthesis in *A. parasiticus* SU1. Measurements of mycelial dry weights in Reddy's and YES growth media were performed essentially as described previously (54). Direct competitive enzyme-linked immunosorbent assay analyses of AFB1 production were performed as described by Pestka (46) with AFB1 monoclonal antibodies and AFB1-horseradish peroxidase conjugate (both kindly provided by J. Pestka, Michigan State University).

Transformation of *A. parasiticus* and genetic disruption of gene-1. Transformation of protoplasts of *A. parasiticus* CS10 was conducted using minor modifications of the polyethylene glycol method (43), as previously described (50). *pyrG*$^+$ prototrophs were selected on Czapek Dox medium (Difco). pAPNVES43 (see Fig. 4) was used as the disruption vector. pPG3J, containing the *pyrG* gene only (50), served as a control plasmid to measure the rate of successful transformation. Transformant clones were transferred to CAM to screen for VA accumulation. Transformant clones were purified by single spore isolation three successive times.

Genetic and biochemical analysis of gene-1 disruptant clones. Ehrlenmeyer flasks (250 ml) containing 100 ml of YES broth were inoculated (10^6 spores) with gene-1 disruptant clones or ATTC 36537 (control; parental strain of transformation recipient strain CS10) and incubated without agitation at 30°C in the dark. After 72 h of growth, mycelial mats were removed and blotted dry. One-quarter (wet weight) of the mycelial mat was dried overnight at 60°C to determine dry weight. The remainder of each mycelial mat and the growth medium were extracted separately with acetone and then with chloroform as previously described (54). TLC analyses of the solvent extracts were performed on activated high-performance silica TLC plates (10 by 10 cm) in a chamber equilibrated with benzene-acetic acid (95:5). Purified VA (generously provided by Deepak Bhatnagar) was resolved on each plate as a standard. DNA was purified from mycelium grown separately in YES broth for 48 h as described above and was analyzed by Southern hybridization.

Sclerotium production. Gene-1 disruptant clones were tested for the ability to produce sclerotia. Aflatoxin-producing strain SU1, NA-accumulating strain ATCC 24690, VA-accumulating strain ATCC 36537, and AVF-accumulating strain ATCC 24551 were grown under identical conditions for comparison. Strains were center inoculated onto petri plates containing approximately 30 ml of CAM medium and incubated for 14 days in the dark at 30°C. Sclerotia were harvested and counted by a published modification (48) of a method previously described by Cotty (19).

Nucleotide sequence analysis. Nucleotide sequence analysis was conducted on cosmid NorA subclones (clones 3 and 4, two *Sac*I-*Eco*RI restriction fragments encoding a large portion of gene-1) at the Plant Research Laboratory at Michigan State University and by DNA Technologies Inc., Rockville, Md. Automated nucleotide sequencers (ABI robotic catalyst and 373A DNA sequencer) and fluorescent labelled T3 and T7 oligonucleotide primers were used to generate and analyze dideoxy sequence reactions. Nucleotide sequence data were analyzed with the Wisconsin Genetics Computer Group Package. The locations of introns and open reading frames were predicted with the software programs Frames, Testcode, and Codon Preference and the *A. nidulans* codon usage file described previously (35, 54). Comparisons of predicted amino acid sequences to EMBL and GenBank database libraries were conducted with TFastA and Gap and aligned with Pileup from the Wisconsin Genetics Computer Group Package.

RESULTS

Restriction endonuclease analysis of cosmid NorA and physical linkage of *nor-1* and *ver-1*. In screening an *A. parasiticus* SU1 genomic DNA cosmid library, four cosmid clones hybridized to the *ver-1* probe (NorA, Ver2, Ver3, and Ver4) and two clones hybridized to the *nor-1* probe (NorA and NorB). Cosmid NorA was of particular interest because it hybridized to both the *nor-1* and *ver-1* gene probes. A restriction endonuclease map of cosmid NorA was generated to allow localization of *nor-1*, *ver-1*, and *uvm8* genes (an *Eco*RI and *Xba*I restriction map is shown in Fig. 1). Since cosmid NorA hybridized to both *nor-1* and *ver-1*, it is suggested that either the two genes are physically linked in the genome of *A. parasiticus* or *nor-1* and *ver-1* were brought together on cosmid NorA due to recombination of normally unlinked chromosomal fragments. To distinguish between these possibilities, Southern hybridization analyses were performed on cosmid NorA and genomic DNA isolated directly from toxigenic *A. parasiticus* SU1 (Fig. 2A). The *nor-1* probe hybridized to identical 22-kb *Xba*I DNA restriction fragments in cosmid NorA and in genomic DNA. The *ver-1* probe hybridized to a 19-kb *Xba*I fragment in cosmid NorA and a 21-kb *Xba*I fragment in genomic DNA. A 3.2-kb *Sac*I-*Bam*HI subclone from cosmid NorA which spanned the junction between the two large *Xba*I fragments (22 and 19 kb) hybridized to the same 22- and 21-kb

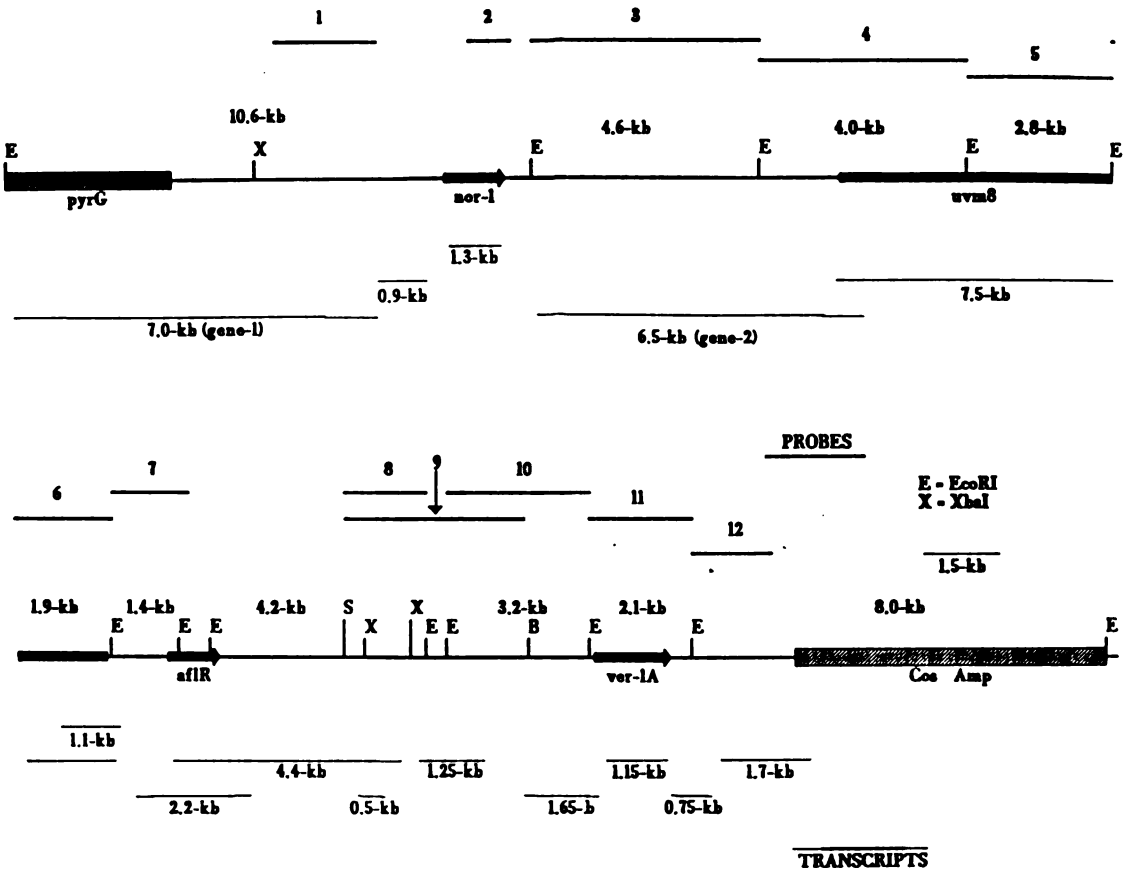

FIG. 1. Restriction endonuclease and transcript map of cosmid NorA. Sizes and locations of transcripts and *Eco*RI restriction fragments are shown. ^{32}P-labelled probes used to locate transcripts (see Fig. 3B) are numbered 1 through 12. Locations of genes and directions of transcription are indicated when information is available. Vector sequences are indicated by shaded blocks. The transcribed region of gene-1 continues beyond the end of cosmid NorA. All *Xba*I (X) and *Eco*RI (E) sites are shown. The locations of *Sac*I (S) and *Bam*HI (B) sites are only included to mark the probe used in linkage analysis; other sites are not included.

*Xba*I fragments in genomic DNA as the *nor-1* and *ver-1* gene probes (and to the 22- and 19-kb *Xba*I fragments in cosmid NorA). These results strongly suggest that the 22- and 21-kb DNA restriction fragments carrying *nor-1* and *ver-1*, respectively, are directly linked in the genome of *A. parasiticus* SU1. Since the *ver-1* and the 3.2-kb *Sac*I-*Bam*HI probes lie within a 12-kb duplication of the region containing *ver-1* and *aflR* in the genome of SU1 (33, 48), additional bands of the predicted size appeared in the genomic DNA analyzed with these probes (*ver-1* probe, 8.9-kb fragment; 3.2-kb *Sac*I-*Bam*HI probe, 8.9- and 6.5-kb fragments; see Fig. 2B for schematic).

Transcript map of cosmid NorA. The appearance of *nor-1* and *ver-1* transcripts occurs simultaneously in *A. parasiticus* SU1 under different growth conditions, suggesting that they are coordinately regulated in part at the transcriptional level (49). Since the two genes were found to be linked on the chromosome, a transcript mapping analysis of this 35-kb region was initiated to determine the size, location, and pattern of expression of other genes in the region. Genes with expression patterns similar to those of *nor-1* and *ver-1* would be studied further because of the potential for direct involvement in AFB1 synthesis. RNA was isolated at distinct time points from mycelia of aflatoxin-producing *A. parasiticus* SU1 grown in YES or Reddy's medium (which induces AFB1 synthesis). The time courses of aflatoxin production and accumulation of mycelial dry mass were qualitatively similar in the two media (Fig. 3C and D). The maximum rate of fungal growth occurred between 18 and 36 h after inoculation, whereas the maximum rate of aflatoxin synthesis occurred between 48 and 72 h, when growth had slowed considerably in a transition between active growth and stationary phase. Radiolabelled DNA probes (numbered 1 to 12 in Fig. 1) were used to analyze RNA isolated at various times during fungal growth in YES or Reddy's medium. Northern analysis identified the size, location, and pattern of accumulation of 14 transcripts in the region encompassed by cosmid NorA (Fig. 3B, Northern analyses; and Fig. 1, transcript map).

Transcript accumulation in Reddy's and YES media. The pattern of expression observed for genes known to be involved in AFB1 biosynthesis (*nor-1*, *ver-1*, and *uvm8*) in Reddy's medium (a chemically defined medium) showed very little transcript accumulation at the 18-h point and a high level of transcript accumulation between 36 and 84 h (Fig. 3A). Eight transcripts in the gene cluster (from left to right in Fig. 1:

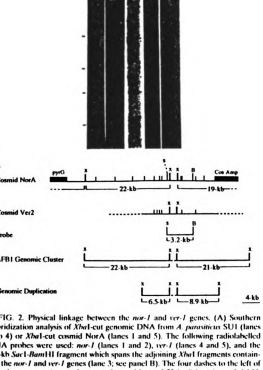

FIG. 2. Physical linkage between the *nor-1* and *ver-1* genes. (A) Southern hybridization analysis of *Xba*I-cut genomic DNA from *A. parasiticus* SU1 (lanes 2 to 4) or *Xba*I-cut cosmid NorA (lanes 1 and 5). The following radiolabelled DNA probes were used: *nor-1* (lanes 1 and 2), *ver-1* (lanes 4 and 5), and the 3.2-kb *Sac*I-*Bam*HI fragment which spans the adjoining *Xba*I fragments containing the *nor-1* and *ver-1* genes (lane 3; see panel B). The four dashes to the left of lane 1 represent (top to bottom) the positions of 23.1-, 9.4-, 6.6-, and 4.4-kb *Hin*dIII restriction fragments of lambda DNA used as a size standard. (B) *Xba*I restriction maps of cosmids NorA and Ver2, and the AFB1 genomic cluster showing the region of genomic DNA duplication. The location of the probe used in panel A is shown. The 19-kb *Xba*I restriction fragment of cosmid NorA is shown on both sides of the 22-kb fragment because of the circular nature of this cosmid. Dashed lines on cosmid Ver2 indicate unmapped regions. Abbreviations are given in the legend to Fig. 1. Unlabelled restriction sites are *Eco*RI sites.

gene-1, 0.9 kb; gene-2, 0.5, 1.25, 1.65, 0.75, and 1.7 kb) showed a pattern of accumulation similar to that of *nor-1*, *ver-1*, and *uvm8*. The data from *nor-1*, *ver-1*, *uvm8*, and gene-1 are shown in Fig. 3A to illustrate the transcript accumulation of these eight genes. In contrast, the accumulation of transcripts for *pyrG*, a gene associated with primary metabolism (UTP biosynthesis), was observed to be high at the 18-h time point. Transcripts of *benA* (encoding β-tubulin) showed nearly uniform accumulation at all time points, including 18 h, as would be expected of a housekeeping gene. The 1.1 and 2.2-kb transcripts (*aflR*) showed transcript accumulation patterns similar to that of *pyrG* (data not shown). It is not known whether the gene encoding the 1.1-kb transcript is involved in secondary metabolism; however, *aflR* has been reported to be a positive regulator of several genes in AFB1 synthesis (55), so the appearance of this transcript before those of *nor-1* and *ver-1* is not unexpected. The 4.4-kb transcript was present in very small quantities at all time points and encompassed the same region of DNA as *aflR*. The nature of this transcript is not clear.

To determine whether the type of growth medium influenced the relative expression of these two different groups of genes (i.e., AFB1-related genes versus primary metabolism or housekeeping genes), the time course of expression in YES, a "rich" AFB1-inducing medium, was also analyzed (Fig. 3B). The contrast between the pattern of expression of *nor-1*, *ver-1*, *uvm8*, and the eight AFB1-related genes and the expression of *pyrG* and *benA* is even more striking. Transcripts of the AFB1-related genes first appeared in the 40-h sample during a transition from active growth to "stationery phase" and decreased significantly by 72 h. In contrast, transcripts of *pyrG* and *benA* were expressed at the highest levels during active growth (first appearing in the 10-h sample) and did not decrease until 24 to 40 h after inoculation, as growth slowed. In both YES and Reddy's media, the appearance of transcripts of AFB1-related genes correlated well with the first appearance of AFB1 in the culture.

Recombinational inactivation of gene-1. The pattern of expression of eight transcripts in cosmid NorA (in addition to *nor-1*, *ver-1*, and *uvm8*) was observed to correlate well with AFB1 synthesis, suggesting that the genes encoding them are involved in AFB1 synthesis. To test this hypothesis, gene-1, encoding a 7-kb transcript (whose function was unknown), was disrupted by insertion of pAPNVES43 (schematic in Fig. 4), which contained an internal fragment of the transcribed region of gene-1. A single-crossover recombinational event between pAPNVES43 and the homologous region of gene-1 in the chromosome should result in insertion of the entire pAPNVES43 vector into gene-1, inactivating its function.

pAPNVES43 was used to transform *A. parasiticus* CS10, a VA-accumulating strain. In two separate experiments, 10% of the *pyrG*$^+$ colonies did not accumulate a yellow pigment (indicative of loss of VA production) on CAM. No transformants were obtained when DNA was not present in the transformation mixture, and no transformants lost their ability to produce the yellow pigment (VA) when pPG3J, carrying only the *pyrG* selectable marker, was used as a control plasmid.

TLC analysis of transformants. Three transformants that no longer appeared to accumulate VA on CAM and a known VA-accumulating strain, ATCC 36537, were grown in YES medium (aflatoxin inducing) for further analysis. TLC analyses of extracts of mycelial mats and the growth medium confirmed a loss of VA production in all three transformed strains (Fig. 5), whereas normal levels of VA were observed in the control strain ATCC 36537 grown under identical conditions. No aflatoxin production was noted in either the transformants or the control strain. No new pigments appeared to accumulate in the putative gene-1-disrupted transformants.

Genetic analysis of putative gene-1 disruptant clones. Southern hybridization analysis was performed on genomic DNA isolated from the parental strain, CS10, and five putative gene-1 disruptants (strains that no longer accumulated VA) (Fig. 6). A 10.2-kb *Eco*RI genomic DNA fragment hybridized to the pUC19 probe (0.8-kb *Sca*I-*Eco*RI), as expected, in four of five transformants (lanes 2, 4, 5, and 6) and to a 3-kb fragment in the fifth transformant (Fig. 6, lane 3; see also schematic in Fig. 4). The occurrence of the 3-kb DNA fragment is likely due to genetic rearrangement during or after integration of the disruption vector. The 10.2-kb fragment was absent in the parental strain (lane 1), as expected. An additional 8-kb fragment was present in two transformants (lanes 2 and 6), indicating that the disruption vector integrated at one other site. Identical DNA samples were also hybridized to a gene-1 probe (0.6-kb *Sma*I-*Sac*I fragment, shown in Fig. 4) located adjacent to the 1.6-kb gene-1 fragment carried on pAPNVES43. The expected 10-kb DNA fragment hybridized to this gene-1 probe in four of the transformed strains (Fig. 6, lanes 8, 10, 11, and 12), indicating insertion of pAPNVES43 by a single crossover at the homologous gene-1 locus on the

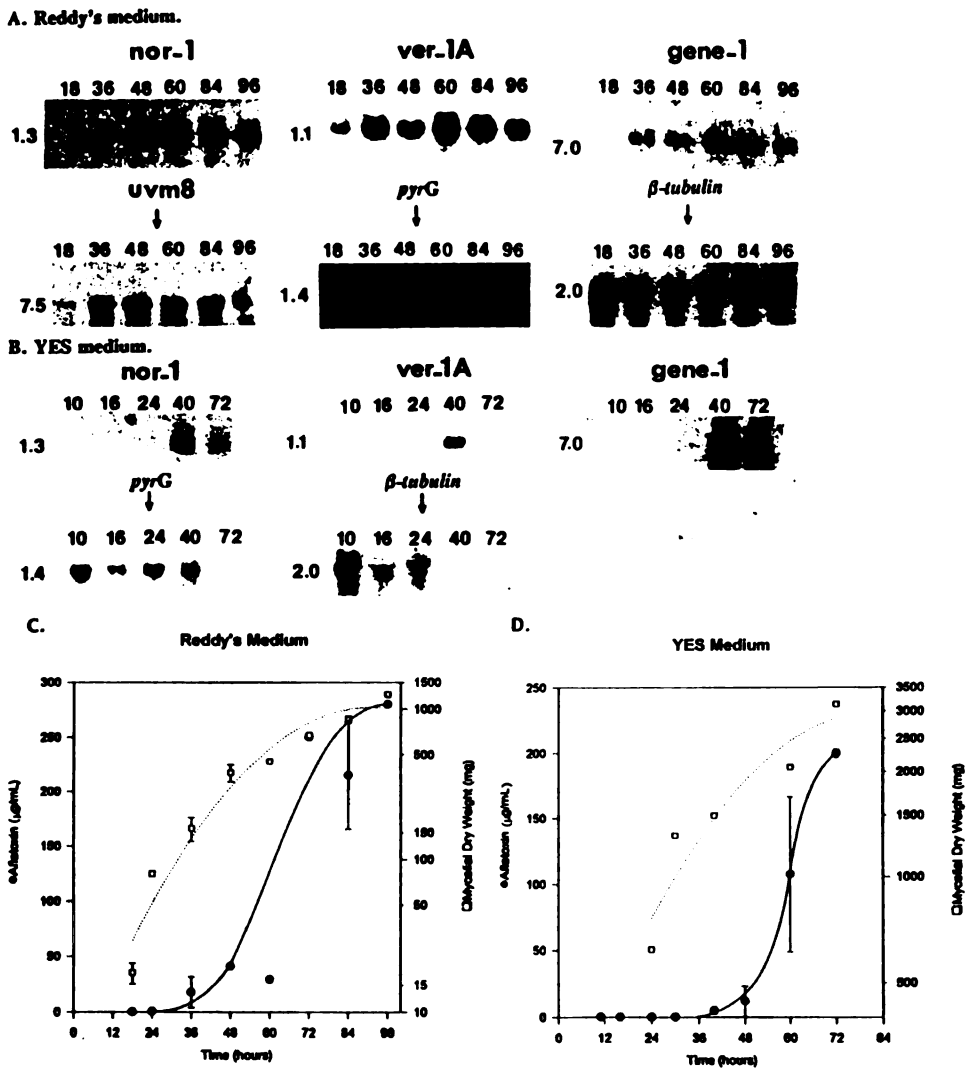

FIG. 3. Accumulation of transcripts of genes in the AFB1 cluster during batch fermentation. *A. parasiticus* NRRL 5862 was inoculated into Reddy's medium and YES medium (time zero) and grown with shaking at 29°C. Samples were removed at the indicated times for extraction of total RNA and analysis of mycelial dry weight and aflatoxin. Northern analyses of the RNA extracted from samples grown in Reddy's (A) and YES (B) media were done with the DNA probes for hybridization shown in Fig. 1 and described in Materials and Methods. Hybridization to *pyrG* and β-tubulin genes (controls) is also shown. Production of aflatoxin and mycelial dry weights are shown in panels C (Reddy's medium) and D (YES medium). Vertical bars indicate standard errors of the mean.

chromosome. The presence of a larger fragment in lane 9 and the absence of a 10-kb fragment support the genetic rearrangement argument proposed for the same DNA sample probed with pUC19 (lane 2). The probe hybridized to the expected 13.2-kb *Eco*RI genomic DNA fragment in CS10. DNA samples were also hybridized to a *pyrG* gene probe, which confirmed that the disruption vector was inserted by a single-crossover event into gene-1 (data not shown). The complete hybridization analysis was repeated with genomic DNA cut with *Eco*RV and *Sac*I (data not shown). These data confirmed the results observed for the *Eco*RI digests.

Sclerotium production. Two gene-1 disruptant clones (Tf1 and Tf2) as well as strains SU1 (aflatoxin accumulating), ATCC 24690 (NA accumulating; small quantities of AFB1 are also produced), ATCC 24551 (AVF accumulating), and ATCC 36537 (VA accumulating) were inoculated onto CAM and grown for 14 days at 30°C. These strains could be divided into three distinct groups on the basis of levels of sclerotia produced (Table 1). Gene-1 disruptants (which do not accumulate AFB1 or identifiable pathway intermediates) produced about three to six times the quantity of sclerotia produced by the wild type, SU1. The NA (an early pathway intermediate)-accumulating strain produced quantities of sclerotia similar to those produced by SU1, while the two strains that accumulated the pathway intermediates VA and AVF (intermediates near the middle of the pathway) and produced

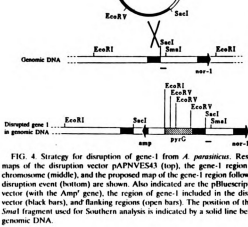

FIG. 4. Strategy for disruption of gene-1 from *A. parasiticus*. Restriction maps of the disruption vector pAPNVES43 (top), the gene-1 region of the chromosome (middle), and the proposed map of the gene-1 region following the disruption event (bottom) are shown. Also indicated are the pBluescriptSK(−) vector (with the Amp^r gene), the region of gene-1 included in the disruption vector (black bars), and flanking regions (open bars). The position of the *Sac*I-*Sma*I fragment used for Southern analysis is indicated by a solid line below the genomic DNA.

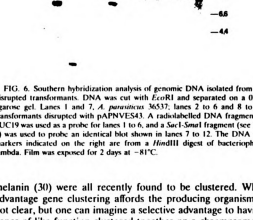

FIG. 6. Southern hybridization analysis of genomic DNA isolated from the disrupted transformants. DNA was cut with *Eco*RI and separated on a 0.8% agarose gel. Lanes 1 and 7, *A. parasiticus* 36537; lanes 2 to 6 and 8 to 12, transformants disrupted with pAPNVES43. A radiolabelled DNA fragment of pUC19 was used as a probe for lanes 1 to 6, and a *Sac*I-*Sma*I fragment (see Fig. 4) was used to probe an identical blot shown in lanes 7 to 12. The DNA size markers indicated on the right are from a *Hin*dIII digest of bacteriophage lambda. Film was exposed for 2 days at −81°C.

no detectable AFB1 failed to produce sclerotia or produced very few sclerotia.

DISCUSSION

Here, evidence is presented that several genes involved in AFB1 biosynthesis (*nor-1*, *ver-1*, *aflR*, *avnA8*, and gene-1) are physically linked on cosmid NorA and in the chromosome of *A. parasiticus* SU1. The *omt-1* gene has also been linked to this cluster of genes in *A. parasiticus* and *A. flavus* (57). Nucleotide sequence analysis of this entire gene cluster in *A. parasiticus* and a structurally similar (but not identical) gene cluster in *A. nidulans* is progressing. DNA sequence analysis of the entire region will allow identification of open reading frames, which may provide clues about the possible function of the seven other AFB1-related genes.

It is not surprising that the aflatoxin genes would be arranged in a cluster in the genome of *Aspergillus* organisms. Many genes involved in secondary metabolism in fungi have been found to be clustered. For example, genes involved in the biosynthesis of penicillin (21, 42), trichothecenes (26), and melanin (30) were all recently found to be clustered. What advantage gene clustering affords the producing organism is not clear, but one can imagine a selective advantage to having genes of like function clustered together on a chromosome if clustering is related to regulation of gene expression.

Recombinational inactivation of gene-1 provides the first indication that this gene is directly involved in aflatoxin biosynthesis and sets a precedent that other genes in the cluster, which are expressed in a pattern similar to those of *nor-1* and *ver-1* (AFB1-related genes), are also prime candidates to be involved in AFB1 synthesis. The activity of the product of gene-1 remains undescribed. However, data from two separate research approaches provide clues about its function. Nucleotide sequence comparisons between the proposed amino acid sequences in two distinct regions of gene-1 and proteins in the EMBL and GenBank database libraries were made, using computer-assisted analyses (Wisconsin Genetics Computer Group; TFastA and MOTIFS). High degrees of similarity (80%) and identity (64%) were observed between a 100-amino-acid domain in the gene-1 protein sequence and the β-ketoacyl-acyl carrier protein-synthase (Fig. 7A) functional domain of the *A. nidulans wA* gene (41), a polyketide synthase gene (PKS) involved in conidial pigment synthesis. The two other proteins that showed high identity in the same region were the *Streptomyces antibioticus* PKS (22) and the *Streptomyces erythrea* PKS (52), with identities of 29.0 and 25.0%, respectively (Fig. 7A). A significant level of identity (20 to 32%) was observed in the acyltransferase functional domains of the

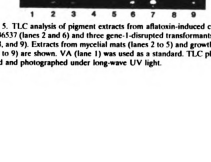

FIG. 5. TLC analysis of pigment extracts from aflatoxin-induced cultures of ATCC 36537 (lanes 2 and 6) and three gene-1-disrupted transformants (lanes 3, 4, 5, 7, 8, and 9). Extracts from mycelial mats (lanes 2 to 5) and growth medium (lanes 6 to 9) are shown. VA (lane 1) was used as a standard. TLC plates were observed and photographed under long-wave UV light.

TABLE 1. Sclerotium production in various strains of *A. parasiticus* grown on CAM for 14 days

Strain[a]	Intermediate accumulating	No. of sclerotia	
		Plate 1	Plate 2
TI1	ND[b]	5,020	3,104
TI2	ND	6,136	5,713
24690	NA	1,242	1,002
24551	AVF	0	0
36537	VA	36	5
SU1	AFB1	983	1,716

[a] Strain 24551 is derived from ATCC 15517 (24); all others are SU1 derivatives.
[b] ND, not detected.

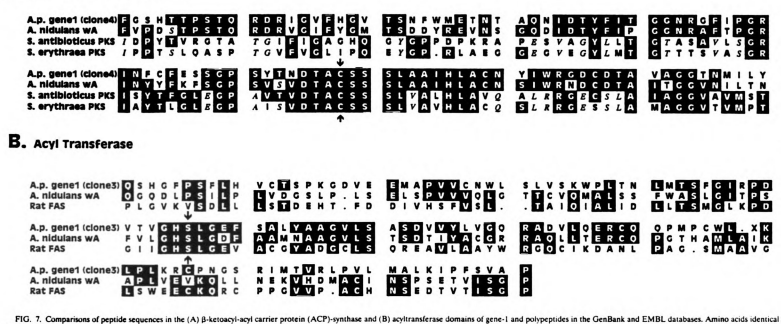

FIG. 7. Comparisons of peptide sequences in the (A) β-ketoacyl-acyl carrier protein (ACP)-synthase and (B) acyltransferase domains of gene-1 and polypeptides in the GenBank and EMBL databases. Amino acids identical in at least two of the presented sequences are shown as white letters on black backgrounds. When two different pairs of identical amino acids occur at the same residue, the pairs which do not match the *A. parasiticus* (A.p.) sequence are shown in italics. Arrows in panels A and B indicate active-site cysteines and serines, respectively. See text for analysis of comparisons.

wA PKS (32%) and rat fatty acid synthase (FAS) (20% [51]) enzymes and a distinct region in the gene-1 sequence (Fig. 7B). The identity of gene-1 in these two functional domains was higher with PKS than with FAS, suggesting that gene-1 encodes a PKS involved in AFB1 synthesis. However, these data do not rule out the possibility that gene-1 encodes an FAS.

Limited nucleotide sequence analysis of *uvm8* (37) identified a 180-amino-acid region with a high degree of sequence identity (48%) to a region of undefined function in FAS1 genes encoding the beta subunit of FAS in the yeasts *Saccharomyces cerevisiae* and *Yarowia lipolytica* (31). Townsend et al. (53) proposed that six-carbon hexanoate (two keto groups completely reduced to hydrocarbon) may serve as the starter unit for AFB1 synthesis and that hexanoate is extended by a PKS without further ketoreduction to form a decaketide, NA. This scheme would include at least one multifunctional enzyme, the FAS, with the necessary activities to reduce keto groups to hydrocarbons in order to synthesize the hexanoate starter. Another set of activities, the PKS, without keto reduction capability (27), would then extend hexanoate to generate the decaketide, NA. Our limited data are consistent with this scheme. *uvm8*, which has a high degree of identity to yeast FAS, could fill the hypothetical FAS role to produce hexanoate, which is extended by the product of gene-1 (*pksA*), a putative PKS. In support of this theory, Chang et al. (14) have independently disrupted and sequenced more extensive regions of gene-1, which they have called *pksA*. Several functional domains associated with polymerization of acetate (β-ketoacyl-synthase, acyltransferase, and acyl carrier protein) were identified, but the analysis strikingly failed to find evidence for a keto-reductase, dehydratase, or enoyl reductase involved in reduction of keto (carbonyl) groups to hydrocarbon.

A second approach, gene disruption, clearly demonstrated that gene-1 activity occurs prior to the *ver-1* gene in the pathway. Further evidence for gene-1 function is provided by the studies on sclerotium production in gene-1-disrupted transformants. The absence of production of sclerotia by strains that accumulate pathway intermediates between NA and VA suggests that gene-1 is involved at a step prior to *nor-1* activity. In a separate study it was shown that strains disrupted at *uvm8*, like strains disrupted in gene-1, produce sclerotia at levels higher than those produced by SU1 (36). Since it was demonstrated that *uvm8* activity occurs before *nor-1* in the pathway, this would argue that gene-1, like *uvm8*, is involved in some stage of polyketide backbone synthesis.

In previous research, an association between aflatoxin biosynthesis and sclerotium development has been observed (6, 18, 19). Using a molecular genetics approach, Skory et al. (48) observed that complementation of the *ver-1* mutation in strain *A. parasiticus* CS10, which accumulates VA and normally does not produce sclerotia on potato dextrose agar, restored wild-type levels of sclerotium production. The data presented here for the AVF- and VA-accumulating strains suggest that accumulation of pathway intermediates inhibits sclerotium development. Strains which accumulate early pathway intermediates (NA) or no pathway intermediates (gene-1 and *uvm8* disruptants) generate wild-type levels of sclerotia (or more), suggesting that elimination of accumulation of intermediates in the middle of the pathway allows sclerotial development to occur. When no intermediates accumulate, sclerotial development is apparently enhanced. Together, these observations support the hypothesis that the biosynthetic pathway for aflatoxin production strongly affects the development of sclerotia. Since secondary metabolism has long been considered a form of metabolic differentiation (7), it is not surprising that it may also be linked to morphological differentiation. Just how this link is structured remains to be elucidated.

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REFERENCES

1. **Anderson, J. A., and L. D. Green.** 1994. Timing of appearance of versiconal hemiacetal acetate esterase and versiconal cyclase activity in cultures of *Aspergillus parasiticus*. Mycopathologia **126**:169–172.
2. **Arseculeratne, S. N., L. M. De Silva, S. Wijesundera, and C. H. S. R. Bandunatha.** 1969. Coconut as a medium for the experimental production of aflatoxin. Appl. Microbiol. **18**:88–94.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology, vol. 1. John Wiley & Sons, Inc., New York.
4. **Bennett, J. W., and L. A. Goldblatt.** 1973. The isolation of mutants of *Aspergillus flavus* and *Aspergillus parasiticus* with altered aflatoxin producing ability. Sabouraudia **11**:235–241.
5. **Bennett, J. W., L. S. Lee, S. M. Shoss, and G. H. Boudreaux.** 1980. Identification of averantin as an aflatoxin B_1 precursor: placement in the biosynthetic pathway. Appl. Environ. Microbiol. **39**:835–839.
6. **Bennett, J. W., P. M. Leong, S. Kruger, and D. Keyes.** 1986. Sclerotial and low aflatoxigenic morphological variants from haploid and diploid *Aspergillus parasiticus*. Experientia **42**:848–851.
7. **Bennett, J. W., and K. E. Papa.** 1988. The aflatoxigenic *Aspergillus* spp. Adv. Plant Pathol. **6**:265–279.
8. **Bhatnagar, D., and T. E. Cleveland.** 1990. Purification and characterization of a reductase from *Aspergillus parasiticus* SRRC 2043 involved in aflatoxin biosynthesis. FASEB J. **4**:2727.
9. **Bhatnagar, D., K. C. Ehrlich, and T. E. Cleveland.** 1992. Oxidation-reduction reactions in biosynthesis of secondary metabolites, p. 255–286. *In* D. Bhatnagar, E. B. Lillehoj, and D. K. Arora (ed.), Handbook of applied mycology, vol. 5. Marcel Dekker, Inc., New York.
10. **Bhatnagar, D., G. Payne, J. E. Linz, and T. E. Cleveland.** 1995. Potential contributions of molecular genetics to aflatoxin elimination. Inform **6**:262–272.
11. **Bhatnagar, D., A. H. J. Ullah, and T. E. Cleveland.** 1988. Purification and characterization of a methyltransferase from *Aspergillus parasiticus* SRRC 163 involved in aflatoxin biosynthetic pathway. Prep. Biochem. **18**:321–349.
12. **Bradshaw, R. E., J. W. Bennett, and J. F. Peberdy.** 1983. Parasexual analysis of *Aspergillus parasiticus*. J. Gen. Microbiol. **129**:2117–2123.
13. **Chang, P.-K., J. W. Cary, D. Bhatnagar, T. E. Cleveland, J. W. Bennett, J. E. Linz, C. P. Woloshuk, and G. A. Payne.** 1993. Cloning of the *Aspergillus parasiticus apa-2* gene associated with the regulation of aflatoxin biosynthesis. Appl. Environ. Microbiol. **59**:3273–3279.
14. **Chang, P.-K., J. W. Cary, J. Yu, D. Bhatnagar, and T. E. Cleveland.** *Aspergillus parasiticus pksA*, a homolog of *Aspergillus nidulans wA*, is required for aflatoxin B_1 biosynthesis. Submitted for publication.
15. **Chang, P.-K., C. D. Skory, and J. E. Linz.** 1992. Cloning of a gene associated with aflatoxin B1 biosynthesis in *Aspergillus parasiticus*. Curr. Genet. **21**:231–233.
16. **Chuturgoon, A. A., M. F. Dutton, and R. K. Berry.** 1990. The preparation of an enzyme associated with aflatoxin biosynthesis by affinity chromatography. Biochem. Biophys. Res. Commun. **166**:38–42.
17. **Cleveland, T. E., and D. Bhatnagar.** 1990. Evidence for de novo synthesis of an aflatoxin pathway methyltransferase near the cessation of active growth and the onset of aflatoxin biosynthesis in *Aspergillus parasiticus* mycelia. Can. J. Microbiol. **36**:1–5.
18. **Cotty, P. J.** 1988. Aflatoxin and sclerotial production by *Aspergillus flavus*: influence of pH. Phytopathology **78**:1250–1253.
19. **Cotty, P. J.** 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology **79**:808–814.
20. **Council for Agricultural Science and Technology (CAST).** 1989. Mycotoxins: economic and health risks. Report 116. Council for Agricultural Science and Technology, Ames, Iowa.
21. **Diez, B., S. Gutierrez, J. L. Barredo, P. van Solingen, L. H. M. van der Voort, and J. F. Martin.** 1990. The cluster of penicillin biosynthetic genes. Identification and characterization of the pcbAB gene encoding the alpha-aminoadipyl-cysteinyl-valine synthetase and linkage to the pcbC and penDE genes. J. Biol. Chem. **265**:16358–16365.
22. **Donadio, S., M. J. Staver, J. B. McAlpine, S. J. Swanson, and L. Katz.** 1991. Modular organization of genes required for complex polyketide biosynthesis. Science **252**:675–679.
23. **Donkersloot, J. A., R. I. Mateles, and S. S. Yang.** 1972. Isolation of averufin from a mutant of *Aspergillus parasiticus* impaired in aflatoxin biosynthesis. Biochem. Biophys. Res. Commun. **47**:1051–1055.
24. **Dutton, M. F.** 1988. Enzymes and aflatoxin biosynthesis. Microbiol. Rev.

52:274–295.
25. **Dvorackova, I.** 1990. Aflatoxins and human health. CRC Press, Boca Raton, Fla.
26. **Hohn, T. M., S. P. McCormick, and A. E. Desjardins.** 1993. Evidence for a gene cluster involving trichothecene-pathway biosynthetic genes in *Fusarium sporotrichioides*. Curr. Genet. 24:291–295.
27. **Hopwood, D. A., and D. H. Sherman.** 1990. Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. Annu. Rev. Genet. 24:39–66.
28. **Keller, N. P., H. C. Dischinger, Jr., D. Bhatnagar, T. E. Cleveland, and A. H. J. Ullah.** 1992. Purification of a 40-kilodalton methyltransferase active in the aflatoxin biosynthetic pathway. Appl. Environ. Microbiol. 59:479–484.
29. **Keller, N. P., N. J. Kantz, and T. H. Adams.** 1994. *Aspergillus nidulans verA* is required for production of the mycotoxin sterigmatocystin. Appl. Environ. Microbiol. 60:1444–1450.
30. **Kimura, N., and T. Tsuge.** 1993. Gene cluster involved in melanin biosynthesis of the filamentous fungus *Alternaria alternata*. J. Bacteriol. 175:4427–4435.
31. **Köttig, H., G. Rottner, K.-F. Beck, M. Schweizer, and E. Schweizer.** 1991. The pentafunctional FAS1 genes of *Saccharomyces cerevisiae* and *Yarrowia lipolytica* are co-linear and considerably longer than previously estimated. Mol. Gen. Genet. 226:310–314.
32. **Lee, L. S., J. W. Bennett, L. A. Goldblatt, and R. E. Landin.** 1970. Norsolorinic acid from a mutant strain of *Aspergillus parasiticus*. J. Am. Oil Chem. Soc. 48:93–94.
33. **Liang, S.-H., and J. E. Linz.** 1994. Structural and functional characterization of the *ver-1* genes and proteins from *Aspergillus parasiticus* associated with the conversion of versicolorin A to sterigmatocystin in aflatoxin biosynthesis. *In* Proceedings, Current issues in food safety. Toxicology Center, Michigan State University, Oct. 1994. Michigan State University, East Lansing.
34. **Lin, B.-K., and J. A. Anderson.** 1992. Purification and properties of versiconal cyclase from *Aspergillus parasiticus*. Arch. Biochem. Biophys. 293:67–70.
35. **Lloyd, A. T., and P. M. Sharp.** 1991. Codon usage in *Aspergillus nidulans*. Mol. Gen. Genet. 230:288–294.
36. **Mahanti, N., D. Bhatnagar, and J. E. Linz.** 1994. Structure and function of *uvm8*, a gene involved in polyketide backbone synthesis of the aflatoxin biosynthetic pathway of *Aspergillus parasiticus*. American Phytopathological Society Meeting, Albuquerque, N.M.
37. **Mahanti, N., F. Trail, R. Mehigh, T. S. Wu, R. Zhou, H. S. Liang, M. Rarick, and J. Linz.** 1993. Structure/function analysis of an aflatoxin gene cluster isolated from *Aspergillus parasiticus*, p. 31. *In* Proceedings, Aflatoxin elimination workshop, Little Rock, Ark. Food Safety and Health, ARS, USDA, Beltsville, Md.
38. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor laboratory, Cold Spring Harbor, N.Y.
39. **Muramatsu, M.** 1973. Preparation of RNA from animal cells. Methods Cell Biol. 7:23–51.
40. **Matsushima, K.-I., Y. Ando, T. Hamasaki, and K. Yabe.** 1994. Purification and characterization of two versiconal hemiacetal acetate reductases involved in aflatoxin biosynthesis. Appl. Environ. Microbiol. 60:2561–2567.
41. **Mayorga, M. E., and W. E. Timberlake.** 1992. The developmentally regulated *Aspergillus nidulans wA* gene encodes a polypeptide homologous to polyketide and fatty acid synthases. Mol. Gen. Genet. 235:205–212.
42. **Montenegro, E., R. Fierro, F. J. Fernandez, S. Gutierrez, and J. F. Martin.** 1992. Resolution of chromosomes III and VI of *Aspergillus nidulans* by pulsed-field gel electrophoresis shows that the penicillin biosynthetic pathway genes *pcbAB*, *pcbC*, and *penDE* are clustered on chromosome VI (3.0 megabases). J. Bacteriol. 174:7063–7067.
43. **Oakley, B. R., J. E. Rinehart, B. L. Mitchell, C. E. Oakley, C. Carmona, G. L. Gray, and G. S. May.** 1987. Cloning, mapping, and molecular analysis of the pyrG (orotidine-5′-phosphate decarboxylase) gene of *Aspergillus nidulans*. Gene 61:385–399.
44. **Papa, K. E.** 1984. Genetics of *Aspergillus flavus*: linkage of aflatoxin mutants. Can. J. Microbiol. 30:68–73.
45. **Payne, G. A., G. J. Nystrom, D. Bhatnagar, T. E. Cleveland, and C. P. Woloshuk.** 1993. Cloning of the *afl-2* gene involved in aflatoxin biosynthesis from *Aspergillus flavus*. Appl. Environ. Microbiol. 59:156–162.
46. **Pestka, J. J.** 1988. Enhanced surveillance of foodborne mycotoxins by immunochemical assay. J. Assoc. Off. Anal. Chem. 71:1075–1081.
47. **Reddy, T. V., L. Viswanathan, and T. A. Venkitasubramanian.** 1971. High aflatoxin production on a chemically defined medium. Appl. Microbiol. 22: 393–396.
48. **Skory, C. D., P.-K. Chang, J. Cary, and J. E. Linz.** 1992. Isolation and characterization of a gene from *Aspergillus parasiticus* associated with the conversion of versicolorin A to sterigmatocystin in aflatoxin biosynthesis. Appl. Environ. Microbiol. 58:3527–3537.
49. **Skory, C. D., P.-K. Chang, and J. E. Linz.** 1993. Regulated expression of the *nor-1* and *ver-1* genes associated with aflatoxin biosynthesis. Appl. Environ. Microbiol. 59:1642–1646.
50. **Skory, C. D., J. S. Horng, J. J. Pestka, and J. E. Linz.** 1990. Transformation of *Aspergillus parasiticus* with a homologous gene (*pyrG*) involved in pyrimidine biosynthesis. Appl. Environ. Microbiol. 56:3315–3320.
51. **Smith, S., J. K. Naggert, B. Williams-Ahlf, and C. M. Amy.** 1992. Intron-exon organization of the gene for the multifunctional animal fatty acid synthase. Proc. Natl. Acad. Sci. USA 89:1105–1108.
52. **Swan, D. G., A. M. Rodriguez, C. Vilches, C. Mendez, and J. A. Salas.** 1994. Characterization of a *Streptomyces antibioticus* gene encoding a type I polyketide synthase which has an unusual coding sequence. Mol. Gen. Genet. 242:358–362.
53. **Townsend, C. A., S. M. McGuire, S. W. Brobst, T. L. Graybill, K. Pal, and C. E. Barry III.** 1991. Examination of tetrahydro- and dihydrobisfuran formation in aflatoxin biosynthesis: from whole cells to purified enzymes, p. 141–154. *In* R. J. Petroski and S. P. McCormick (ed.), Secondary-metabolite biosynthesis and metabolism. Plenum Press, Inc., New York.
54. **Trail, F., P.-K. Chang, J. Cary, and J. E. Linz.** 1994. Structural and functional analysis of the *nor-1* gene involved in the biosynthesis of aflatoxins by *Aspergillus parasiticus*. Appl. Environ. Microbiol. 60:4078–4085.
55. **Woloshuk, C. P., K. R. Foutz, J. F. Brewer, D. Bhatnagar, T. E. Cleveland, and G. A. Payne.** 1994. Molecular characterization of *aflR*, a regulatory locus for aflatoxin biosynthesis. Appl. Environ. Microbiol. 60:2408–2414.
55a. **Wu, T.-S., and J. E. Linz.** Unpublished data.
56. **Yabe, K., Y. Matsuyama, Y. Ando, H. Nakajima, and T. Hamasaki.** 1993. Stereochemistry during aflatoxin biosynthesis: conversion of norsolorinic acid to averufin. Appl. Environ. Microbiol. 59:2486–2492.
57. **Yu, J., J. W. Cary, D. Bhatnagar, T. E. Cleveland, N. P. Keller, and F. S. Chu.** 1993. Cloning and characterization of a cDNA from *Aspergillus parasiticus* encoding an *O*-methyltransferase involved in aflatoxin biosynthesis. Appl. Environ. Microbiol. 59:3564–3571.
58. **Yu, J., P.-K. Chang, J. W. Cary, M. Wright, D. Bhatnagar, T. E. Cleveland, G. A. Payne, and J. E. Linz.** 1995. Comparative mapping of aflatoxin pathway gene clusters in *Aspergillus parasiticus* and *Aspergillus flavus*. Appl. Environ. Microbiol. 61:2365–2371.

In the following manuscript I was primarily responsible for the sequence analysis and figures generated from that work. I also helped with the photography of the thin layer chromatography plates.

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Structure and Function of *fas-1A*, a Gene Encoding a Putative Fatty Acid Synthetase Directly Involved in Aflatoxin Biosynthesis in *Aspergillus parasiticus*

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A novel gene, *fas-1A*, directly involved in aflatoxin B1 (AFB1) biosynthesis, was cloned by genetic complementation of an *Aspergillus parasiticus* mutant strain, UVM8, blocked at two unique sites in the AFB1 biosynthetic pathway. Metabolite conversion studies localized the two genetic blocks to early steps in the AFB1 pathway (*nor-1* and *fas-1A*) and confirmed that *fas-1A* is blocked prior to *nor-1*. Transformation of UVM8 with cosmids NorA and NorB restored function in *nor-1* and *fas-1A*, resulting in synthesis of AFB1. An 8-kb *Sac*I subclone of cosmid NorA complemented *fas-1A* only, resulting in accumulation of norsolorinic acid. Gene disruption of the *fas-1A* locus blocked norsolorinic acid accumulation in *A. parasiticus* B62 (*nor-1*), which normally accumulates this intermediate. These data confirmed that *fas-1A* is directly involved in AFB1 synthesis. The predicted amino acid sequence of *fas-1A* showed a high level of identity with extensive regions in the enoyl reductase and malonyl/palmityl transferase functional domains in the beta subunit of yeast fatty acid synthetase. Together, these data suggest that *fas-1A* encodes a novel fatty acid synthetase which synthesizes part of the polyketide backbone of AFB1. Additional data support an interaction between AFB1 synthesis and sclerotium development.

Aflatoxins are polyketide-derived secondary metabolites that are produced by strains of the imperfect fungi *Aspergillus parasiticus* and *Aspergillus flavus*. Aflatoxins are highly toxic, mutagenic, and carcinogenic in a variety of animal species and are suspected carcinogens in humans (11). Peanuts, treenuts, corn, cottonseed, and other important crops are occasionally contaminated with aflatoxin as a result of infection by toxigenic aspergilli. An understanding of the aflatoxin biosynthetic pathway may result in the identification of strategies to inhibit aflatoxin contamination of plant-derived products at the preharvest level.

Aflatoxin biosynthesis is proposed to begin with the condensation of acetyl coenzyme A and malonyl coenzyme A via a polyketide synthetase (PKS) to form the decaketide noranthrone (4, 10). Alternatively, a six-carbon fatty acid, hexanoate, is first synthesized by a fatty acid synthetase (FAS) and then extended by a PKS to generate noranthrone (22). Noranthrone is oxidized to norsolorinic acid (NA), which is converted to aflatoxin B1 (AFB1) through a series of pathway intermediates, including averantin (AVN), averufanin, averufin, versiconal hemiacetal acetate, versiconal, versicolorin B, versicolorin A (VA), demethylsterigmatocystin, sterigmatocystin (ST), *o*-methylsterigmatocystin (OMST), and AFB1 (4, 10).

Several genes encoding enzyme activities or regulatory proteins involved in AFB1 biosynthesis in *A. parasiticus* and *A. flavus* and ST biosynthesis in *Aspergillus nidulans* have been cloned (6, 24). These genes are clustered in a 65-kb region on one chromosome in *A. parasiticus* and *A. flavus* (25, 26). Transcript mapping analysis identified three other genes in the cluster encoding large transcripts (7.5, 7.0, and 6.5 kb) which appear to be involved in AFB1 biosynthesis (25). Gene disruption and nucleotide sequence analyses of *pksA* (7.0-kb transcript) suggested that it encodes a PKS involved in synthesis of the AFB1 polyketide backbone (7, 25).

This study focuses on *fas-1A*, which encodes the 7.5-kb transcript. Analysis of *fas-1A* mutants combined with nucleotide sequence analysis strongly suggests that this gene encodes one subunit of a novel FAS directly involved in synthesis of the AFB1 polyketide backbone.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. Plasmid DNA was propagated in *Escherichia coli* DH5α (12) and purified by an alkaline lysis procedure (18). Plasmid pRZ2.8 (see Fig. 3) contains a 2.8-kb *Eco*RI subclone of cosmid NorA inserted into the *Eco*RI site of plasmid pBluescriptIISK(−). Plasmid pAPSa8 (Fig. 1) contains an 8-kb *Sac*I subclone of cosmid NorA inserted into the *Sac*I site of pBluescriptIISK(−).

A. parasiticus SU1 (ATCC 56775, NRRL 5862) served as the aflatoxin-producing wild-type strain. *A. parasiticus* B62 (*niaD nor-1 bi-1* [8]), derived from *A. parasiticus* ATCC 24690 (16), was used for isolation of mutants created by UV mutagenesis and for gene disruption experiments. B62 accumulates NA and retains the ability to produce low levels of AFB1 (five- to eightfold less than SU1).

Methods for the maintenance of fungal strains and preparation of conidial stocks and descriptions of the liquid and solid growth media used for production (YES) and analysis (coconut agar medium [CAM] [2]) of aflatoxins have been reported previously (25).

UV mutagenesis. Conidia in sterile water (10^6 per ml) were exposed to up to 10 sequential doses of UV light at 200,000 μJ per dose (UV Stratalinker; Stratagene). Mutants lacking NA synthesis were obtained after irradiation with seven doses (UVM7) or eight doses (UVM8).

Metabolite conversion studies. Metabolite conversion studies with whole cells were conducted as described by Bhatnagar et al. (5) and Adye and Mateles (1). One gram (wet weight) of washed mycelia from UVM7 or UVM8 was incubated for 12 h with constant shaking (150 rpm) at 28°C in the presence of acetate (1,000 μg), NA (10 μg), AVN (10 μg), VA (10 μg), ST (5 μg), or OMST (5 μg). Aflatoxins were analyzed by thin-layer chromatography (TLC) and quantitated by densitometry (Shimadzu dual-wavelength TLC scanner model CS9000U) as described by Bhatnagar et al. (5).

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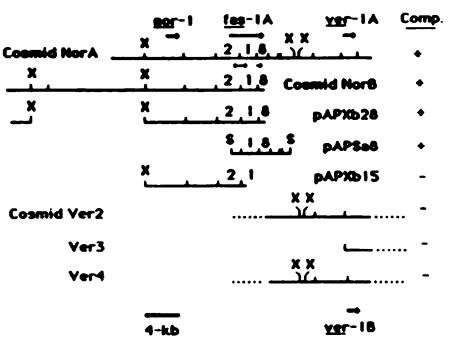

FIG. 1. DNA fragments used for complementation of UVM8. Seven DNA fragments, represented by solid lines, were used in complementation experiments. Only insert DNA is shown. Complementation (Comp.) of UVM8 is shown for each fragment. Unlabeled restriction endonuclease sites are *Eco*RI. Other sites are *Sac*I (s) and *Xba*I (x). The thick arrows represent the size and orientation of transcripts from *nor-1*, *ver-1A*, *ver-1B*, and *fas-1A*. The numbers 2, 1, and 8 on the maps for cosmids NorA and NorB and subclones pAPXb28 and pAPSa8 are *Eco*RI subclones used in nucleotide sequence analysis. The small arrows on the NorA map show the approximate length and direction of sequencing to generate the data shown in Fig. 6. The dotted lines on the maps of cosmids Ver2, -3, and -4 represent unmapped regions.

Genetic complementation: transformation of fungal protoplasts and analysis of transformant clones. Protoplasts were transformed by a polyethylene glycol procedure (21). Plasmid pSL82 contains the nitrate reductase gene *niaD* (13), used as a selectable marker for cotransformation. The cosmids used in complementation experiments (Fig. 1) were isolated in a previous study (25): cosmid NorA contains *nor-1* and *ver-1A* (17); cosmid NorB contains *nor-1* on a 21-kb DNA fragment that overlaps with NorA; and cosmids Ver2, Ver3, and Ver4 contain all or part of a 12-kb duplication of the *ver-1A aflR* region on cosmid NorA. *ver-1B*, a nearly identical copy of *ver-1A*, is contained in this duplicated region (17). Cosmids or subclones were added in 2- to 10-fold molar excess over pSL82. *niaD*$^+$ transformants, selected by growth on Czapek Dox (CZ) medium, were transferred to CAM to screen for aflatoxin and NA accumulation.

Analyses of aflatoxin production in transformants by TLC and ELISA. Aflatoxin and NA produced by the recipient strain B62 and *fas-1A* disruptants were quantitated by TLC and enzyme-linked immunosorbent assay (ELISA) by the method of Trail et al. (23) except that cells were cultured for 65 h instead of 72 h.

Genomic DNA isolation and Southern analysis. Genomic DNA was prepared by a published modification (14) of a phenol-chloroform protocol developed for mammalian DNA (3). Restriction endonucleases were purchased from New England Biolabs or Boehringer Mannheim Biochemicals. ^{32}P-labeled DNA probes were generated with the Random Primed DNA Labelling Kit from Boehringer Mannheim Biochemicals. Southern hybridization analyses were conducted by standard procedures (3).

Sclerotium production. Approximately 10^4 conidia were center inoculated onto petri plates containing 20 ml of CAM. Plates were incubated in the dark at 28°C for 7 days. Sclerotia were harvested and counted by a modification (19) of the method of Cotty (9). Sclerotial diameters were measured with a Java video analysis system (Jandal Corp., Corte Madera, Calif.).

TABLE 1. Conversion of metabolites to AFB1 by whole cells of two mutant strains of *A. parasiticus*

Metabolite added	Amt added (μg)	Mean AFB1 produced[a] (μg/mg of mycelium [wet wt])	
		UVM7	UVM8
None		ND	ND
Acetate	1,000	ND	ND
NA	10	0.39 ± 0.06	0.23 ± 0.08
AVN	10	2.1 ± 0.6	1.6 ± 0.4
VA	10	3.4 ± 0.9	4.1 ± 0.6
ST	5	3.1 ± 0.2	2.8 ± 0.4
OMST	5	4.6 ± 1.0	5.1 ± 0.8

[a] Mean of two experiments with two replicates each. ND, none detected.

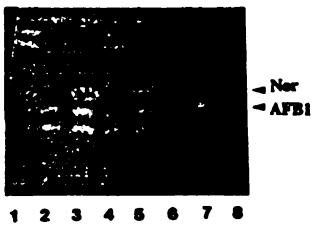

FIG. 2. TLC analysis of cell extracts from UVM8 complementation experiments. Lanes: 1, UVM8; 2, UVM7; 3, B62; 4, UVM8 transformed with pAPXb28; 5 and 6, two UVM8 isolates transformed with pAPSa8; 7, NA standard (arrow labeled Nor); 8, AFB1 standard (arrow labeled AFB1). The band immediately below AFB1 in lanes 2, 3, 4, 5, and 6 is AFG1.

Nucleotide sequence analyses. Nucleotide sequence analyses were conducted by DNA Technologies, Inc., Gaithersburg, Md., on three cosmid NorA subclones containing *fas-1A* (clones 2, 1, and 8 [Fig. 1]) (25). Nucleotide sequence data were analyzed with the Wisconsin Genetics Computer Group (GCG) software package. The locations of introns and open reading frames were predicted by using GCG programs Frames, TestCode, and CodonPreference. Comparisons of the predicted amino acid sequence of *fas-1A* with sequences in the EMBL and GenBank databases were conducted with TFastA and Gap.

Nucleotide sequence accession number. The accession number for *fas-1A* is L48183.

RESULTS

Isolation of UV mutants UVM7 and UVM8. UVM7 and UVM8, derived from *A. parasiticus* B62, no longer accumulated NA (red-orange pigment) or AFB1 (blue fluorescence) on CAM. UVM7 produced nonpigmented mycelia, and UVM8 produced a bright yellow mycelial pigment that was secreted into the growth medium. Loss of AFB1 and NA synthesis was confirmed by TLC analysis (data not shown). Inability to grow on CZ medium indicated that these mutants retained a nonfunctional *niaD* allele.

Metabolite conversion studies. UVM7 and UVM8 converted VA, AVN, ST, and OMST to AFB1 but could not convert acetate to NA or AFB1 (Table 1), suggesting that they were blocked prior to *nor-1* (the product of *nor-1* converts NA

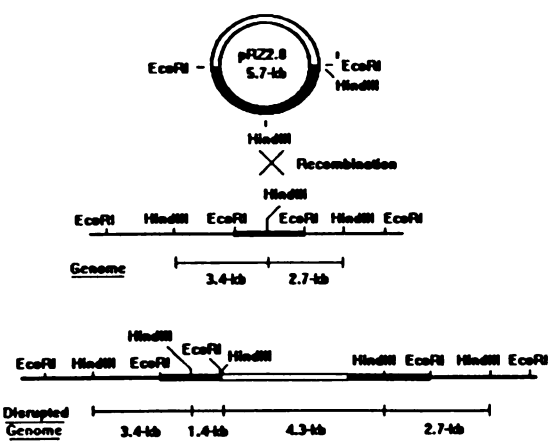

FIG. 3. Gene disruption of *fas-1A*. Recombination between the 2.8-kb insert in pRZ2.8 (solid area) and the homologous region in *fas-1A* (solid box) in the genome generates *Hin*dIII restriction fragments of the sizes indicated on the map labeled disrupted genome (the probe is the 2.8-kb insert). Nondisrupted strains have *Hin*dIII fragments of the sizes indicated on the map labeled genome.

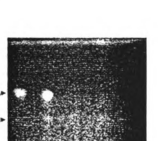

FIG. 4. TLC analysis of cell extracts from Dis1, -2, and -3. Lanes: 1, NA standard; 2, extract from B62; 3, 4, and 5, extracts from Dis1, -2, and -3, respectively.

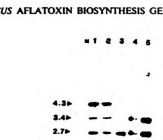

FIG. 5. Southern hybridization analysis of Dis1, -2, and -3. Genomic DNAs, digested with *Hin*dIII, were subjected to Southern hybridization analysis with the 2.8-kb *Eco*RI insert from pRZ2.8 as a probe. Arrowheads show the sizes of expected hybridizing fragments (in kilobases). Lanes: M, molecular size markers; 1, 2, and 3, genomic DNA isolated from Dis1, -2, and -3, respectively; 4 and 5, genomic DNA from *niaD*⁻; NA-accumulating transformants (nondisrupted strains).

to AVN). The mutants converted five- to eightfold more AVN to AFB1 than NA, suggesting that they retained the *nor-1* genotype and were therefore blocked at two sites in the AFB1 pathway, *nor-1* and *fas-1A*.

Complementation of UVM8. UVM8 was cotransformed with plasmid pSL82 plus one of five cosmids or cosmid subclones (Fig. 1). Cosmids NorA and NorB complemented both pathway mutations in approximately 1% of the *niaD*⁺ transformants in two separate experiments, resulting in synthesis of AFB1. Cosmids Ver2, Ver3, and Ver4 and plasmids pAPXb15, containing a 15-kb subclone of cosmid 69R (8), and pSL82 (control) failed to complement UVM8.

Cloning of *fas-1A*. A 28-kb *Xba*I subclone of cosmid NorB (pAPXb28 [Fig. 1]), which carried the 21-kb overlap between these clones, complemented *fas-1A* and *nor-1* or *fas-1A* alone in strain UVM8, resulting in transformants which produced AFB1 (6 of 160 *niaD*⁺ transformants) or NA plus small quantities of AFB1 (five- to eightfold less than SU1) (1 of 160 *niaD*⁺ transformants), respectively. Comparison of the restriction endonuclease maps of cosmids NorA and NorB and plasmids pAPXb28 and pAPXb15 localized *fas-1A* to three contiguous *Eco*RI subclones of cosmid NorA (clones 2, 1, and 8 [Fig. 1]). An 8-kb *Sac*I subclone of cosmid NorA (pAPSa8) containing clones 1 and 8 and part of clone 2 was used with plasmid pSL82 to cotransform strain UVM8. Two of 30 *niaD*⁺ transformants accumulated NA on CAM, suggesting that pAPSa8 complemented *fas-1A* (and not *nor-1*) in UVM8. Control transformants (pSL82 only) did not produce NA on CAM.

TLC analysis of the recipient strain and transformed isolates (Fig. 2) determined that UVM8 failed to produce detectable AFB1 or NA, whereas UVM8 transformed with pAPXb28 produced AFB1. UVM8 transformed with pAPSa8 produced NA and AFB1 at levels similar to those of the *nor-1* mutant *A. parasiticus* 24690. UVM7 produced low levels of AFB1 and aflatoxin G1 (AFG1), suggesting that the mutations in UVM7 and UVM8 are not allelic.

Gene disruption of *fas-1A*. Transcript mapping analysis previously localized a 7.5-kb transcript to the *fas-1A* locus (25) (Fig. 1). A 2.8-kb *Eco*RI cosmid NorA subclone (pRZ2.8 [Fig. 3]) from the middle of the 7.5-kb coding region was used to disrupt *fas-1A* in strain B62, an NA-accumulating strain. Approximately 4% of the *niaD*⁺ transformants failed to produce detectable NA or AFB1 when grown on CAM, suggesting that they were *fas-1A* disruptants. All pSL82 transformants (control) produced NA on CAM. No transformants were obtained in the absence of plasmid DNA.

Three putative *fas-1A* disruptant clones, Dis1, Dis2, and Dis3, were subjected to TLC (Fig. 4) and direct competitive ELISA analyses. Dis1, -2, and -3 did not produce detectable NA or AFB1, whereas B62 transformed with pSL82 (control) accumulated NA and low levels of AFB1. Direct competitive ELISA confirmed that B62 transformed with pSL82 (control) produced 300-fold more AFB1 (3 to 5 μg/ml) than Dis1, Dis2, and Dis3 (0.002 to 0.015 μg/ml; near the limit of detection). Strain SU1 (wild type) produced approximately 6,000-fold more AFB1 (100 μg/ml) than Dis1, -2, and -3 and approximately 20-fold more AFB1 than strain B62.

The disruption of *fas-1A* in Dis1, -2, and -3 was confirmed by Southern hybridization analysis with the ^{32}P-labeled 2.8-kb *Eco*RI fragment of pRZ2.8 as a probe (Fig. 5). Genomic DNA isolated from Dis1 and Dis2 contained the expected 1.4- and 4.3-kb *Hin*dIII fragments (Fig. 3) in addition to the 3.4-kb and 2.7-kb fragments present in the recipient strain B62. Dis3 contained only the 2.7-kb DNA fragment, suggesting that genetic recombination between tandem copies of *fas-1A* resulted in the deletion of part of both copies of *fas-1A* plus the vector sequences in between. The deletion was not due to a precise excision of pRZ2.8 because the 3.4-kb *Hin*dII fragment was deleted and because *fas-1A* remained nonfunctional. The same filter was reprobed with ^{32}P-labeled pBluescript11SK(−) (data not shown), which hybridized to a 4.2-kb *Hin*dIII fragment, as expected, in Dis1 and Dis2 but not in Dis3, consistent with the hypothesized deletion event. The vector DNA hybridized to a 2.8-kb *Hin*dIII fragment in all three disruptants, as expected, because of the integration of pSL82 (which contains pUC19) at the *niaD* locus. Southern hybridization analyses on identical

TABLE 2. Sclerotium production in *A. parasiticus*

Strain[a]	No. of sclerotia[b]	Avg diam[b] (μm)
B62	600	470 ± 130
SU1 (Afl⁺)	2,400	470 ± 90
Dis1	3,000	490 ± 140
Dis2	2,100	460 ± 100
Dis3	5,400	460 ± 100

[a] B62 is the recipient strain used in the *fas-1A* disruption experiment. SU1 is an aflatoxin-producing wild-type strain. Dis1, -2, and -3 are three *fas-1A* disruptants selected for further study.
[b] Means of two experiments.

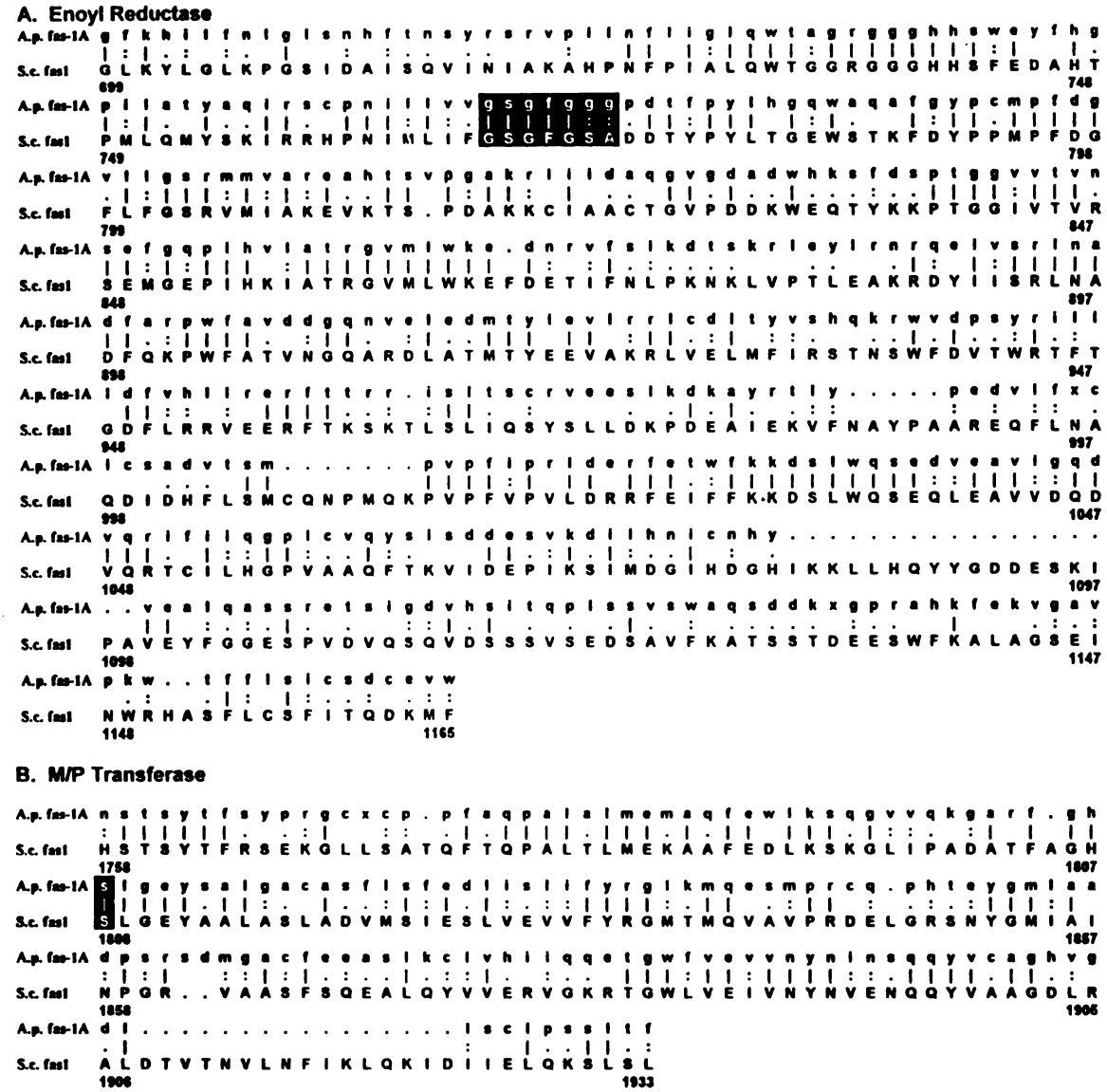

FIG. 6. Gap (GCG) comparison of *A. parasiticus fas-1A* and *S. cerevisiae FAS1* products (15). Predicted amino acid sequences encoded in two regions of *fas-1A* were compared with functional domains in the yeast *FAS1* gene product by using the GCG software Gap. (A) Gap analysis of the enoyl reductase functional domain. The putative active-site motif is highlighted. (B) Gap analysis of malonyl-palmityl (M/P) transferase functional domain. The putative active-site residue (serine) is highlighted. Numbers in the yeast amino acid sequence are those reported by Kottig et al. (15). Vertical lines between residues in the comparison represent identity, two dots represent more highly conserved substitutions, and a single dot represents less highly conserved substitutions.

genomic DNAs digested with *Sca*I confirmed the *Hin*dIII data (not shown).

Sclerotium development. Dis1 and Dis2 produced four- to fivefold and Dis3 produced approximately ninefold more sclerotia than B62 (Table 2). The number of sclerotia produced by the aflatoxin-producing strain SU1 was similar to that produced by Dis1 and Dis2; however, Dis3 produced twofold more sclerotia than SU1.

DISCUSSION

Because *fas-1A* is necessary for synthesis of NA, this argues that *fas-1A* encodes either noranthrone oxidase (10) or an activity involved in polyketide backbone synthesis. The large size of the *fas-1A* transcript suggested that it might encode a multifunctional protein, similar to *pksA* (7, 25). Nucleotide sequence analysis was conducted on two extensive regions of *fas-1A* to determine if predicted amino acid sequence data

might provide clues about *fas-1A* function (Fig. 6). Comparison of the predicted amino acid sequence of the *fas-1A* product with proteins in the GenBank and EMBL databases with the TFastA program detected a high level of identity with FAS1 proteins from *Saccharomyces cerevisiae* and *Yarrowia lipolytica* (15). *FAS1* encodes the beta subunit of FAS, a protein which contains four functional domains typical of FASs, including (from amino terminus to carboxyl terminus) acetyltransferase, enoyl reductase, dehydratase, and malonyl/palmityl transferase (15). A 435-amino-acid region in the *fas-1A* product displayed 40% identity and 58% similarity with the enoyl reductase domain in FAS1, while a 159-amino-acid region displayed 47% identity and 69% similarity to the malonyl/palmityl transferase domain (including the active-site residues). These two domains appeared in the same relative position and order in the *fas-1A* product as in FAS1. These data strongly suggest that *fas-1A* encodes the beta subunit of a yeast-like FAS1 and support our new designation for this gene, *fas-1A* (formerly *uvm8*).

Townsend et al. (22) proposed that hexanoate, a six-carbon fatty acid, was the starting molecule for polyketide synthesis because NA, the first stable intermediate in AFB1 synthesis, contains a six-carbon "tail" in which two keto groups are completely reduced to hydrocarbon. Hexanoate was proposed to be extended to noranthrone, without further ketoreduction, by a PKS. Our data support this model; the *fas-1A* product is proposed to synthesize hexanoate (or a similar fatty acid starter unit), while the *pksA* product extends hexanoate to noranthrone.

Trail et al. (25) reported that the *fas-1A* transcript accumulates under aflatoxin-inducing conditions with the same pattern as *nor-1* and *ver-1* (20), suggesting that *fas-1A*, like *nor-1* and *ver-1*, is involved in secondary metabolism. Disruption of *fas-1A* in the current study had no apparent effect on the growth of *A. parasiticus* on CZ, a defined minimal growth medium that contains no added fatty acids. Together, the data suggest that *fas-1A* is involved in AFB1 synthesis and not in the synthesis of fatty acids required for growth.

Disruption of *fas-1A* also enhanced sclerotium development compared with the parental strain B62, a phenotype similar to *pksA* disruptants (25). Since no AFB1 pathway intermediates accumulate in Dis1, Dis2, or Dis3 or *pksA* disruptants, the accumulation of certain pathway intermediates (i.e., NA, AVN, and VA) appears to downregulate sclerotium development. This hypothesis is supported by previous observations. Strain CS10 (*ver-1 wh-1 pyrG*), which accumulates VA (an intermediate near the middle of the AFB1 pathway), produces few sclerotia on CAM at 30°C (19). Complementation of CS10 with *ver-1* restores wild-type levels of AFB1 synthesis and sclerotium production. The nature of the interaction between AFB1 synthesis and sclerotium development remains unclear and deserves further study.

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REFERENCES

1. **Adye, J., and R. I. Mateles.** 1964. Incorporation of labelled compounds into aflatoxins. Biochim. Biophys. Acta **86**:418–420.
2. **Arseculeratne, S. N., L. M. De Silva, S. Wijesundera, and C. H. S. R. Bandunatha.** 1969. Coconut as a medium for the experimental production of aflatoxin. Appl. Microbiol. **18**:88–94.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology, vol. 1. John Wiley & Sons, Inc., New York.
4. **Bhatnagar, D., K. C. Ehrlich, and T. E. Cleveland.** 1992. Oxidation-reduction reactions in biosynthesis of secondary metabolites, p. 255–286. *In* Handbook of applied mycology, vol. 5, mycotoxins in ecological systems. Marcel Dekker, New York.
5. **Bhatnagar, D., S. P. McCormick, L. S. Lee, and R. A. Hill.** 1987. Identification of *O*-methylsterigmatocystin as an aflatoxin B_1 and G_1 precursor in *Aspergillus parasiticus*. Appl. Environ. Microbiol. **53**:1028–1033.
6. **Bhatnagar, D., G. Payne, J. E. Linz, and T. E. Cleveland.** 1995. Molecular biology to eliminate aflatoxins. Inform **6**:262–274.
7. **Chang, P.-K., J. W. Cary, J. Yu, D. Bhatnagar, and T. E. Cleveland.** *Aspergillus parasiticus pksA*, a homolog of *Aspergillus nidulans wA*, is required for aflatoxin B_1 biosynthesis. Mol. Gen. Genet., in press.
8. **Chang, P.-K., C. D. Skory, and J. E. Linz.** 1992. Cloning of a gene associated with aflatoxin B1 biosynthesis in *Aspergillus parasiticus*. Curr. Genet. **21**:231–233.
9. **Cotty, P. J.** 1988. Aflatoxin and sclerotial production of *Aspergillus flavus*: influence of pH. Phytopathology **78**:1250–1253.
10. **Dutton, M. F.** 1988. Enzymes and aflatoxin biosynthesis. Microbiol. Rev. **52**:274–295.
11. **Dvorackova, I.** 1990. Aflatoxins and human health. CRC Press, Boca Raton, Fla.
12. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
13. **Horng, J.-S., P.-K. Chang, J. J. Pestka, and J. E. Linz.** 1990. Development of a homologous transformation system for *Aspergillus parasiticus* with the gene encoding nitrate reductase. Mol. Gen. Genet. **224**:294–296.
14. **Horng, J.-S., J. E. Linz, and J. J. Pestka.** 1989. Cloning and characterization of the *trpC* gene from an aflatoxigenic strain of *Aspergillus parasiticus*. Appl. Environ. Microbiol. **55**:2561–2568.
15. **Kottig, H., G. Rottner, K. Beck, M. Schweizer, and E. Schweizer.** 1991. The pentafunctional FAS1 genes of *Saccharomyces cerevisiae* and *Yarrowia lipolytica* are co-linear and considerably longer than previously estimated. Mol. Gen. Genet. **226**:310–314.
16. **Lee, L. S., J. W. Bennett, L. A. Goldblatt, and R. E. Lundin.** 1970. Norsolorinic acid from a mutant strain of *Aspergillus parasiticus*. J. Am. Oil Chem. Soc. **48**:93–94.
17. **Liang, S.-H., and J. E. Linz.** 1994. Structural and functional characterization of the *ver-1* genes and proteins associated with the conversion of versicolorin A to sterigmatocystin in aflatoxin biosynthesis, poster 20. *In* Proceedings: Current Issues in Food Safety. Toxicology Center, Michigan State University, East Lansing, Mich.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Skory, C. D., P.-K. Chang, J. Cary, and J. E. Linz.** 1992. Isolation and characterization of a gene from *Aspergillus parasiticus* associated with conversion of versicolorin A to sterigmatocystin in aflatoxin biosynthesis. Appl. Environ. Microbiol. **58**:3527–3537.
20. **Skory, C. D., P.-K. Chang, and J. E. Linz.** 1993. Regulated expression of the *nor-1* and *ver-1* genes associated with aflatoxin biosynthesis. Appl. Environ. Microbiol. **59**:1642–1646.
21. **Skory, C. D., J. S. Horng, J. J. Pestka, and J. E. Linz.** 1990. A transformation system for *Aspergillus parasiticus* based on a homologous gene involved in pyrimidine biosynthesis (*pyrG*). Appl. Environ. Microbiol. **56**:3315–3320.
22. **Townsend, C. A., S. M. McGuire, S. W. Brobst, T. L. Graybill, K. Pal, and C. E. Barry III.** 1991. Examination of tetrahydro- and dihydrobisfuran formation in aflatoxin biosynthesis: from whole cells to purified enzymes, p. 141–154. *In* R. J. Petroski and S. P. McCormick (ed.), Secondary-metabolite biosynthesis and metabolism. Plenum Press, New York.
23. **Trail, F., P.-K. Chang, J. W. Cary, and J. E. Linz.** 1994. Structural and functional analysis of the *nor-1* gene involved in the biosynthesis of aflatoxins by *Aspergillus parasiticus*. Appl. Environ. Microbiol. **60**:4078–4085.
24. **Trail, F., N. Mahanti, and J. E. Linz.** 1995. Review—molecular biology of aflatoxin biosynthesis. Microbiology **141**:755–765.
25. **Trail, F., N. Mahanti, M. Rarick, R. Mehigh, S.-H. Liang, R. Zhou, and J. E. Linz.** 1995. A physical and transcriptional map of an aflatoxin gene cluster in *Aspergillus parasiticus* and the functional disruption of a gene involved early in the aflatoxin pathway. Appl. Environ. Microbiol. **61**:2665–2673.
26. **Yu, J., P.-K. Chang, J. W. Cary, M. Wright, D. Bhatnagar, T. E. Cleveland, G. A. Payne, and J. E. Linz.** 1995. Comparative mapping of aflatoxin pathway gene clusters in *Aspergillus parasiticus* and *Aspergillus flavus*. Appl. Environ. Microbiol. **61**:2365–2371.

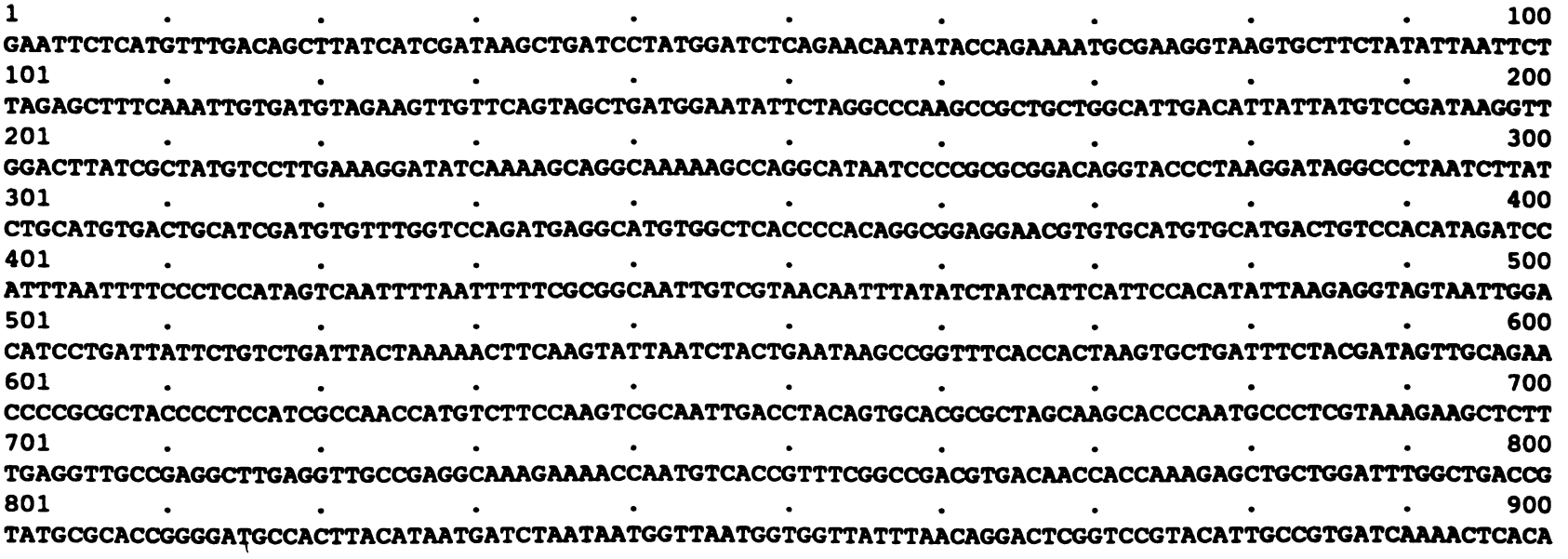
APPENDIX B

Appendix B - Sequence analysis of the *A. parasiticus pyr*G

The absence of a sexual cycle in certain species has made the production of mutants and subsequent isolation of genes a difficult prospect. Several *Aspergillus* spp. including *A. parasiticus* and *A. flavus* fall into this category. Fortunately, transformation systems have been developed which allow the introduction of naked DNA to 1) complement mutations of interest and 2) restore the function of a marker gene. Two common marker systems use the complementation of mutant genes encoding nitrate reductase (*nia*D) (Wu and Linz, 1993), and orotidine 5'-monophosphate decarboxylase (*pyr*G). The *pry*G is responsible for the conversion of orotidine 5'-monophosphate to uridine monophosphate during pyrimidine biosynthesis (Skory, 1992). Auxotrophic strains require media supplemented with uridine to grow. Without it genomic replication cannot proceed. Transformation of these strains with a functional *pyr*G results in conversion to prototrophy. This particular marker system is useful because it is efficient. Skory reported that he was able to obtain 30 to 50 stable transformants per µg DNA with no background growth (Skory et al, 1990). This transformation system has been used in many species including *A. nidulans* (Oakley et al, 1987), *A. niger* (Wilson et al, 1988), *A. flavus* (Woloshuk et al, 1989), *A. parasiticus* (Skory et al, 1990), *S. cerevisiae* (Rose et al, 1984), *Neuropora crassa* (Glazebrook et al, 1987), and *Penicillium chrysogenum* (Cantoral et al, 1988). The nucleotide sequences of *pyr*G from each of these species have been published with the exceptions of *A. flavus* and *A. parasiticus*. By sequencing the third clone of the cosmid NorA (by DNA Technologies) 2700 base pairs of *A. parasiticus* *pyr*G was determined (Figure 19). Subsequent alignment of the *pyr*G genes at the amino

acid level is presented in Figure 20. The identity between *A. parasiticus pyr*G and those from the other species ranged from 78% with *A. nidulans* down to 38% with *N. crassa*. Also, the proposed intron common to all of the published *pyr*G sequences appears to be present in this sequence as well.

Figure 19. Nucleotide sequence of the *A. parasiticus pyr*G.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101 200
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201 300
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401 500
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501 600
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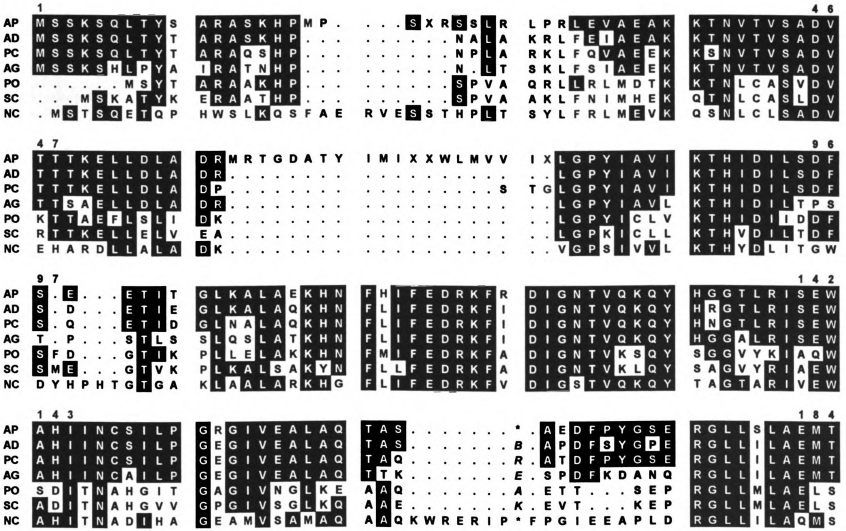
Figure 19.

Figure 20. Amino acid comparison of the *A. parasiticus pyr*G (AP) with homologous genes from A. nidulans (AD, 78% identity), P. chrysogenum (PC, 74% identity), A. niger (AG, 66% identity), P. ohmeri (PO, 43% identity), S. cerevisiae (SC, 43%) and N. crassa (NC, 38% identity)..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AG MSSKSHLPYA IRATNHP... ......N.LT SKLFSIAEEK KTNVTVSADV
PO .....MSYT ARAAKHP... ......SPVA QRLLRLMDTK KTNLCASVDV
SC ...MSKATYK ERAATHP... ......SPVA AKLFNIMHEK QTNLCASLDV
NC .MSTSQETQP HWSLKQSFAE RVESSTHPLT SYLFRLMEVK QSNLCLSADV
47
96
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PC TTTKELLDLA DP........ ....S TGLGPYIAVI KTHIDILSDF
AG TTSAELLDLA DR........ ....LGPYIAVL KTHIDILTPS
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SC RTTKELLELV EA........ ....LGPKICLL KTHVDILTDF
NC EHARDLLALA DK........ ....VGPSIVVL KTHYDLITGW
97
142
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PC S..Q...ETID GLNALAQKHN FLIFEDRKFI DIGNTVQKQY HNGTLRISEW
AG T..P...STLS SLQSLATKHN FLIFEDRKFI DIGNTVQKQY HGGALRISEW
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143
184
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AD AHIINCSILP GEGIVEALAQ TAS.....B. APDFSYGPE RGLLILAEMT
PC AHIINCSILP GEGIVEALAQ TAQ.....R. ATDFPYGSE RGLLILAEMT
AG AHIINCAILP GEGIVEALAQ TTK.....E. SPDFKDANQ RGLLILAEMT
PO SDITNAHGIT GAGIVNGLKE AAQ.....A. ETT...SEP RGLLMLAELS
SC ADITNAHGVV GPGIVSGLKQ AAE.....K. EVT...KEP RGLLMLAELS
NC AHITNADIHA GEAMVSAMAQ AAQKWRERIP*FPGIEEAPLD RGLLILAQMS

Figure 20.

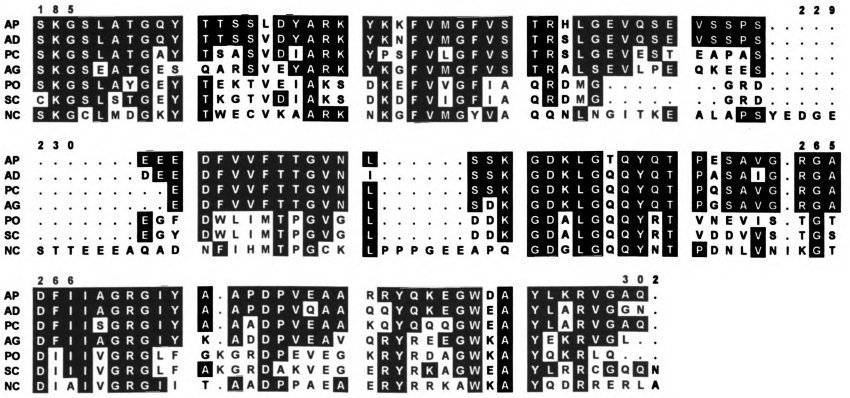
1 8 5
2 2 9
AP SKGSLATGQY TTSSLDYARK YKKFVMGFVS TRHLGEVQSE VSSPS
AD SKGSLATGQY TTSSVDYARK YKNFVMGFVS TRSLGEVQSE VSSPS
PC SKGSLATGAY TSASVDIARK YPSFVLGFVS TRSLGEVEST EAPAS
AG SKGSEATGES QARSVEYARK YKGFVMGFVS TRALSEVLPE QKEES
PO SKGSLAYGEY TEKTVEIAKS DKEFVVGFIA QRDMG GRD
SC CKGSLSTGEY TKGTVDIAKS DKDFVIGFIA QRDMG GRD
NC SKGCLMDGKY TWECVKAARK NKGFVMGYVA QQNLNGITKE ALAPSYEDGE
2 3 0
2 6 5
AP EEE DFVVFTTGVN L SSK GDKLGTQYQT PESAVG RGA
AD DEE DFVVFTTGVN I SSK GDKLGQQYQT PASAIG RGA
PC E DFVVFTTGVN L SSK GDKLGQQYQT PQSAVG RGA
AG E DFVVFTTGVN L SDK GDKLGQQYQT PGSAVG RGA
PO EGF DWLIMTPGVG L DDK GDALGQQYRT VNEVIS TGT
SC EGY DWLIMTPGVG L DDK GDALGQQYRT VDDVVS TGS
NC STTEEEAQAD NFIHMTPGCK LPPPGEEAPQ GDGLGQQYNT PDNLVNIKGT
2 6 6
3 0 2
AP DFIIAGRGIY A.APDPVEAA RRYQKEGWDA YLKRVGAQ.
AD DFIIAGRGIY A.APDPVQAA QQYQKEGWEA YLARVGGN.
PC DFIISGRGIY A.AADPVEAA KQYQQQGWEA YLARVGAQ.
AG DFIIAGRGIY K.ADDPVEAV QRYREEGWKA YEKRVGL..
PO DIIIVGRGLF GKGRDPEVEG KRYRDAGWKA YQKRLQ...
SC DIIIVGRGLF AKGRDAKVEG ERYRKAGWEA YLRRCGQQN
NC DIAIVGRGII T.AADPPAEA ERYRRKAWKA YQDRRERLA

Figure 20.

APPENDIX C

Appendix C - Protocols used in promoter studies

The identification of regulator genes such as *afl*-R is one key in the successful utilization of a genetic approach for eliminating aflatoxins from the food chain. This, however, is only part of the battle. It is necessary to identify how the regulators work. Do they act in a positive or negative manner? Do they act at one site or several? Because the aflatoxin pathway is regulated at least in part at the level of transcription it is reasonable to assume that there are regulatory factors involved in some aspect of transcription. It may also be safe to say that this regulation occurs at the promoter region of one or more genes in the pathway. Studies investigating the proteins that bind promoters of known aflatoxin genes may identify key regulators of the pathway. These studies not only would attemp to identify the factors involved but they would also define the specific sequences they bind. To do these kinds of studies the polymerase chain reaction, nuclear extraction of proteins, gel retardation, and DNAase foot printing, will be employed. The following are modified procedures for performing the above procedures with the exception of DNAase foot printing.

Extraction of Nuclear Proteins from *Aspergillus parasiticus*

Purpose: Extract proteins from the nucleus of *Aspergillus parasiticus* including possible transcription factors involved in aflatoxin biosynthesis.

Modified from Timberlake (1986) and Nagata et al (1993).

Materials

All materials should be autoclaved and/or treated as indicated prior to use.

250ml GSA bottles

SS34 tubes

Spatula and/or rubber policeman

10ml pipettes

4L filter flask(s)

Buchner funnels (1 or 2 for a 4L flask, 1 or 2 for a 1L flask)

Glass beads

Mira cloth

Cheese cloth

The following reagents should be stored at 4°C or kept on ice while being used.

Distilled water

10XSSE salts: 1.0M KCl, 0.1M EDTA, 0.1M Tris base. Make stock of this and autoclave. Just prior to using add Spermidine to 0.04M and Spermine to 0.04M. Adjust pH to 7 with HCl.

1.0XSSE salts (made just prior to use): Combine 100ml of 10XSSE salts with 1ml β-mercaptoethanol, 171.2g Sucrose, and 10ml of PMSF (0.1M in 95% ETOH). Bring to one liter.

Nuclear Extraction Buffer: 10% glycerol, 0.015M Hepes-KOH (pH7.9), 0.5M KCl, 0.005M MgCl2, 0.5mM EDTA. Make a stock of this and autoclave. Just prior to using add 0.001M DTT, 0.5mM PMSF, and two of the following (to 10μg/ml each) antipain, chymostatin, leupeptin, and/or pepstatin.

Dialysis Buffer (prepare just prior to using): 15% glycerol, 0.015M Hepes-KOH (pH7.9), 0.1M KCl, 0.001M EDTA, 0.002M DTT, 0.5mM PMSF, and two of the following (to 10μg/ml each) antipain, chymostatin, leupeptin, and/or pepstatin.

Method

1. Inoculate four 1L flasks containing 500ml medium* with $5x10^8$ spores each.
2. Incubate the flasks 46 hours in the dark at 29°C while shaking at 150 rpm.
3. Harvest the mycelia by vacuum filtration through one layer of mira cloth in a 4L Buchner funnel. Wash the mycelia with chilled water and vacuum dry.
4. Remove the mycelia from the 4L Buchner funnel and place into a 250ml GSA bottle.
5. Add 1.0XSSE and mix with a spatula or rubber policeman until a slurry is formed.
6. Using the bead beater. Fill the bead beater container half to two thirds full with glass beads‡. The actual amount depends on the volume of the slurry. The more the slurry the less the beads (note: the container must be at least half full of beads). Add the slurry. Add 1.0XSSE until the bead beater container is full. Insert blades and secure with collar. Add ice to the collar and blend as follows: blend 45 sec, set 30 sec, blend 45 sec, set 30 sec, blend 30 sec, set until beads settle to bottom of container.
7. Gravity filter the slurry using a 1L Buchner funnel containing four layers of mira cloth covered by two layers of cheese cloth°. The supernatant should be collected in a 250ml GSA bottle which is surrounded by ice. To speed up this filtration two 1L Buchner funnels may be used.
8. Rinse beads in the bead beater container. Add 1.0XSSE to the bead beater container. Insert blades and secure with collar (ice is not necessary). Run bead beater for five to 10 seconds. Let the beads settle. Add the slurry to the 1L Buchner funnel(s) from the

previous step.

9. Centrifuge the GSA bottle(s) at 8000rpm (using the GSA rotor) for 20 minutes at 4°C.

10. Discard the supernatant. Resupend the pellet in 30ml 1.0XSSE using a spatula or rubber policeman.

11. Transfer the suspension to Oakridge tubes (SS34 tubes). Centrifuge at 8200rpm for 20 minutes at 4°C. The point of this centrifugation is to obtain a pellet and a clear supernatant by repeating this step as many times as it takes. It may be more correct to say that performing this centrifugation a total of three times is best. This is due to the fact that sometimes the first centrifugation will yield a clear supernatant, whereas other times a clear supernatant is not obtained even after four centrifugations. Because of these inconsistencies and trial and error three times should suffice. After each centrifugation decant the supernatant and resuspend the pellet in 1.0XSSE.

12. Resuspend pellet in five to 10ml of nuclear extraction buffer. Put tubes in ice and rock on platform shaker for 30 minutes.

13. Transfer suspension to ultracentrifuge tubes (for rotor T865.1). Centrifuge at 36,500rpm for 60 minutes.

14. Decant the supernatant to 15ml conical centrifuge tubes by puncturing the ultracentrifuge tubes with a high gauge needle. To get the entire supernatant out of the tube it may be necessary to carefully (so as not to disturb the pellet) cut the top of the tube off with a pair of scissors.

15. Transfer the extract to dialysis tubing (molecular weight cutoff 3500).

16. Dialyze in dialysis buffer overnight.

17. Decant tubing into SS34 tubes. Centrifuge at 11,200rpm to pellet debris

(clarification).

18. Transfer supernatant to a Centriprep 10 concentrator (Amicon). The instructions with these concentrators say to centrifuge approximately 45 minutes. This does not take into account the temperature (4°C) and the glycerol content of this sample. Because of the lower temperature and higher glycerol content used in this procedure the times will be tremendously increased. This concentrator is only capable of handling 15ml at a time. You will need to centrifuge using a GSA rotor at 4300rpm (3000xg) for a couple hours. At that time add more of the extract and continue centrifugation. Continue to do this until all of the extract has been added. Centrifuge until approximately one or two ml of extract remains. This may take an overnight centrifugation.

19. Determine the concentration of the extract using the protein concentration assay from Biorad.

20. Store extracts at -80°C.

*Possible media are Glucose Minimal Salts (GMS) used to induce aflatoxin production. Peptone Minimal Salts (PMS) used to supress aflatoxin production. Nitrate Minimal Salts (NMS) used to delay aflatoxin production. NMS is the same as GMS except the sole nitrogen source is $NaNO_3$.

‡The glass beads are prepared by soaking in 0.5M HCl (to clean) then rinsing with deionized water (to remove HCl). The beads are ready to be dried when the pH of the rinse water reaches approximately 7.0.

°The mira cloth and the cheese cloth used in this step are prepared by boiling in EDTA three times followed by boiling in distilled water four times then dried.

Polymerase Chain Reaction

Purpose: Amplify specific segments of DNA which are suspected of serving as promoter regions for aflatoxin genes.

The following is a list of reagents used in the reaction mixture for PCR.

Reagent	Volume/sample
DEPC water	(To 50μL)
$MgCl_2$ (25mM)	6μL
10XPCR buffer II	5μL
dNTP mix (10mM)	4μL
Primer 1 (200ng/μL)	1μL
Primer 2 (200ng/μL)	1μL
Taq Polymerase (5units/μL)	1μL
DNA template (200ng)	?
Total volume	50μL

These samples are then placed in a thermocycler (Perkin Elmer) and subject to the following conditions.

-Hold at 95°C for five minutes.

-Cycle the samples 30 times through

95°C for one minute

60°C for one minute

72°C for three minutes

-Hold at 72°C for 10 minutes

-Hold at 4°C until removed

(This may be replaced by Qiagen method) The products are then electrophoresed on a 0.8% agarose gel (containing ethidium bromide) followed by excision of the bands and subsequent purification. These purified products are end labelled using the Ready-to-go labelling kit (Pharmacia Biotech) and γ-^{32}P ATP. Finally, these products are used in mobility shift DNA-binding assays.

Mobility Shift DNA-Binding Assay Using Gel Electrophoresis

Purpose: To determine if a fragment of DNA is bound by protein(s) from a nuclear protein extract.

From Current Protocols (Ausubel et al, 1987).

REFERENCES

List of References

Ausubel, F.M., R. Brent, R.E. Kingstone, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.

Autrup, H., F.J.M. Essigmann, R.G. Croy, B.F. Trump, G.N. Wogan, and C.C. Harris. 1979. Metabolism of aflatoxin B_1 and idnetification of the major aflatoxin B_1-DNA adducts formed in cultured human bronchus and colon. Cancer Res. 39: 694-698.

Ball, R.W., D.W. Wilson, R.A. Coulombe. 1990. Comparative formation and removal of aflatoxin B_1-DNA adducts in cultured mammalian tracheal epithelium. Cancer Res. 50: 4918-4922.

Bennett, J.W., and L.A. Goldblatt. 1973. The isolation of mutants of *Aspergillus flavus* and *Aspergillus parasiticus* with altered aflatoxin producing ability. Sabouraudia. 11: 235-241.

Bhatnagar, D., K.C. Ehrlich, and T.E. Clevelend. 1992. Oxidation-reduction in biosynthesis of secondary metabolites, p.255-286. *In* D. Bhatnagar, E. B. Lillehoj, and D. K. Arora (eds.), Handbook of applied mycology, vol.5: Mycotoxins in ecological systems. Marcel Dekker, Inc. New York.

Bibb, M.J., S. Biro, H. Motamedi, J.F. Collins, and C.R. Hutchinson. 1989. Analysis of the nucleotide sequence of the Streptomyces glaucescens tcm1 genes provides key information about the enzymology of polyketide antibiotic biosynthesis. EMBO J. 8: 2727-2736

Blanco, G., P. Brian, A. Pereda, C. Mendez, J.A. Salas and K.F. Chater. 1993. Hybridization and DNA sequence analyses suggest an early evolutionary divergence of related biosynthetic gene sets encoding polyketide antibiotics and spore pigments in *Streptomyces* spp. Gene. 130:107-116.

Bossche, H.V., D.W.R. Mackenzie, and G. Cauwenbergh. 1987. *Aspergillus* and Aspergillosis. Plenum Press, New York.

Bressac, B., M. Kew, J. Wands, and M. Ozturk. 1991. Selective G to T mutations of the *p53* gene in hepatocellular carcinoma from Southern Africa. Nature. 350: 429-431.

Buchi, G., and I.D. Rae. 1969. The structure and chemistry of the aflatoxins, *In* L.A. Goldblatt (ed.), Aflatoxins. Academic Press, New York.

Bullerman, L.B. 1979. Significance of mycotoxins to food safety and human health. J. Food Prot. 42: 65.

Cantoral, J.M., Barredo, J.L., Diez, B., and Martin, J.F. 1988. Nucliotide sequence of the *Pennecillium chrysogenum pyr*G (orotidine-5'- monophosphate decarboxylase) gene. Nucleic Acids Res. 16: 8177.

CAST (Council for Agricultural Science and Technology). 1979. Mycotoxins: Economic and health risks. Library of Congress Task force report.

CAST (Council for Agricultural Science and Technology). 1989. Mycotoxins: Economic and health risks. Library of Congress Task force report.

Chang, P.-K., J.W. Carry, J. Yu, D. Bhatnagar, and T.E. Cleveland. *Aspergillus parasiticus* pksA, a homologue of *Aspergillus nidulans wA,* is required for aflatoxin B_1 biosynthesis. Appl. Environ. Microbiol. submitted.

Chang, P.-K., J.W. Cary, D. Bhatnagar, T.E. Cleveland, J.W. Bennett, J.E. Linz, C.P. Woloshuk, and G.A. Payne. 1993. Cloning of the *Aspergillus parasiticus apa-2* gene associated with the regulation of aflatoxin biosynthesis. Appl. Environ. Microbiol. 59: 3273-3279.

Chang, P.-K., C.D. Skory, and J.E. Linz. 1992. Cloning of a gene associated with aflatoxin B_1 biosynthesis in *Aspergillus parasiticus*. Curr. Genet. 21: 231-233.

Cole, R.J, R.A. Hill, P.D. Blankenship, T.H. Sanders, and K.H. Garren. 1982. Influence of irrigation and drought stress on invasion of *Aspergillus flavus* of corn kernels and peanut pods. Dev. Ind. Microbiol. 23: 229-236.

Cole, R.J, J.W. Dorner, P.D. Blankenship, and B. Horn. 1991. The use of biocompetitive agents for preharvest aflatoxin control in peanuts. USDA ARS Aflatoxin Elimination Workshop, Atlanta, GA.

Cotty, P.J., P. Bayman, D.S. Egel, and K.S. Elias. 1994. Agriculture, aflatoxins and *Aspergillus*. In: The Genus *Aspergillus*, pp. 1-27, Powell, K.A. et al. Plenum Press, New York.

Cotty, P.J. 1988. Aflatoxin and sclerotia production by *Aspergillus flavus*: influence of pH. Phytopathology. 78: 1250-1253.

Cotty, P.J. 1990. Effect of atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of developing cottonseed. Plant Dis. 74: 233-235.

Croy, R.G., and G.N. Wogan. 1981. Quantitative comparison of covalent aflatoxin-DNA adducts formed in rat and mouse livers and kidneys. J. natn. Cancer Inst. 66: 761-768.

Diener, U.L., R.J. Cole, T.H. Sanders, G.A. Payne, L.S. Lee, and M.A. Klich. 1987. Epidemiology of aflatoxin formation by *Aspergillus flavus*. Ann. Rev. Phytopath. 25: 249-270.

Dorner, J.W., R.J. Cole, and P.D. Blankenship. 1992. Use of a biocompetitive agent to control preharvest aflatoxin in drought stressed peanuts. J. Food Prot. 55: 888-892.

Dvorackova, I. 1990. Aflatoxins and Human Health. CRC Press, Boca Raton.

Eaton, D.L., and J.D. Groopman. 1994. The Toxicology of aflatoxins: human health, veterinary, and agricultural significance. Academic Press, San Diego.

Ehrilch, K. 1987. Effect on aflatoxin production of competition between wild-type and mutant strains of *Aspergillus parasiticus*. Mycopathologia. 97: 93-96.

Ellis, W.O., J.P. Smith, B.K. Simpson, and J.H. Oldham. 1991. Aflatoxins in food: Occurrence, biosynthesis, effects on organisms, detection, and methods of control. Crit. Rev. Food Sci. Nutr. 30: 403-439.

Essigmann, J.M., R.G. Croy, R.A. Bennett, and G.N. Wogan. 1982. Metabolic activation of aflatoxin B_1: pattern of DNA adduct formation, removal, and excretion in relation to carcinogenesis. Drug Metab. Rev. 13: 581-602.

Glazebrook, J.A., K. Mitchell, and A. Radford. 1987. Molecular genetic analysis of the *pyr*-4 gene of *Neurospora crassa*. Mol. Gen. Genet. 209: 399-402.

Gurtoo, H.L. and C.V. Dave. 1975. In vitro metaboic conversion of aflatoxins and benzo(a)pyrene to bucleic acid-binding metabolites. Cancer Res. 35: 382-389.

Harrison, J.C. and R.C. Garner. 1991. Immunological and HPLC detection of aflatoxin adducts in human tissues after an acute poisoning incident in S.E. Asia. Carcinogenesis. 12: 741-743.

Hopwood, D.A., and D.A. Sherman. 1990. Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. Annu. Rev. Genet. 24: 37-66.

Horn, B.W., J.W. Dorner, R.L. Greene, P.D. Blankenship, and R.J. Cole. 1994. Effect of *Aspergillus parasiticus* soil inoculum on invasion of peanut seeds. Mycopathologia. 125: 179-191.

Horng, J.S., P.-K. Chang, J.J. Pestka, and J.E. Linz. 1990. Development of a homologous transformation system for *Aspergillus parasiticus* with the gene encoding nitrate reductase. Appl. Environ. Microbiol. 55: 2561-2568.

Hsieh, D.P.H. The role of aflatoxin in human cancer. In: Mycotoxins and Phycotoxins,

pp. 447-456, Steyn, P.S. and Vleggaar, R. (eds) Elsevier Scientific Publishers, Amsterdam.

Hsieh, D.P.H. Mode of action of mycotoxins. In: Mycotoxins in Food, pp. 149-176, Krogh, P. (ed.) Academic Press, Cambridge

Hsu, I.C., R.A. Metcalf, T. Sun, J.A. Welsh, N.J. Wang, and C.C. Harris. 1991. Mutational hotspot in the *p53* gene in human heptocellular cancinomas. Nature. 350: 427-428.

Jong, S.C., and M.J. Edwards. 1991. American Type Culture Collection Catalogue of Filamentous Fungi, 18th ed. American Type Culture Collection, Rockville, MD.

Keller, N.P., S. Segner, D. Bhatnagar, and T.H. Adams. 1995. stcS, a putative P-450 monooxygenase, is required for the conversion of versicolorin A to sterigmatocystin in Aspergillus nidulans. Appl. Environ. Microbiol. 61: 3628-3632.

Kurtzman, C.P., M.J. Smiley, C.J. Robnett, and D.T. Wicklow. 1986. DNA relatedness among wild and dometicated species in the *Aspergillus flavus* group. Mycologia. 78: 955-959.

Kurtzman, C.P., B.W. Horn, and C.W. Hesseltine. 1987. *Aspergillus nomius,* a new afiatoxin-producing species related to *Aspergillus flavus* and *Aspergillus tamarii.* Antonie Leeuwenhoek. 53:147-158.

Liang, S.-H., and J.E. Linz. 1994. Structural and functional characterization of the *ver*-1 genes and proteins from *Aspergillus parasiticus* associated with the conversion of versicolorin A to demethylsterigmatocystin in aflatoxin biosynthesis. Proceedings of Current Issues in Food Safety. National Food Safety Toxicology Center, Michigan State University, October 1994.

Mahanti, N., D. Bhatnagar, J.W. Cary, J. Joubran, and J.E. Linz. 1996. Structure and Function of *fas*-1A, a gene encoding a putative fatty acid sythetase directly involved in aflatoxin biosynthesis in *Aspergillus parasiticus*.

Mandal, S., A. Ahuja, N.M. Shivapurkar, S.J. Cheng, J.D. Groopman, and G.D Stoner. 1987. Inhibition of aflatoxin B_1 mutagenesis in *Salmonella typhimurium* and DNA damage in cultured rat and human tracheobronchial tissues by ellagic acid. Carcinogenesis. 8: 1651-1656.

Maniatis, T., E.F. Fritsch, and J. Sambrook. 1989. Molecular Cloning: a laboratory manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y.

Maplestone, R.A., M.J. Stone, and D.H. Williams. 1992. The evolutionary role of secondary metabolites-a review. Gene. 15: 151-157.

Maramatsu, M. 1973. Preparation of RNA from animal cells. Methods Cell Biol. 7:23-51.

Massey, T.E., R.K. Stewart, J.M. Daniels, and L. Liu. 1995. Biochemical and molecular aspects of mammalian susceptibility to aflatoxin B_1 carcinogenicity. PSEBM. 208: 213-227.

McLean, M., and M.F. Dutton. 1995. Cellular interactions and metabolism of aflatoxin: an update. Pharmac. Ther. 65: 163-192.

Micheli, P.A. 1729. Nova pantarum genera. Florentiae.

Mohamed, A.H., S.S. Chirala, N.H. Mody, W.-Y. Huang, and S.J. Wakil. 1988. Primary structure fo the multifunctional alpha subunit protein of yeast fatty acid synthase derived from FAS2 gene sequence. J. Biol. Chem. 263: 12315-12325.

Moreau, C. and M. Moss, Eds., Moulds, Toxins and Food, pp. 43, John Wiley and Sons, Chichester, 1979.

Nagata, O., T. Takashima, M. Tanaka, and N. Tsukagoshi. 1993. *Aspergillus nidulans* nuclear proteins bind to a CCAAT element and the adjacent upstream sequence in the promoter region of the starch-inducible Taka-amylase A gene. Mol. Gen. Genet. 237: 251-260.

Neal, G.E., and J.A. Green. 1983. The requirement for glutathione S-transferase in the conjugation of activated aflatoxin B_1 during aflatoxin hepatocarcinogenesis in the rat. Chem. Biol. Interact. 45: 259-275.

Oakley, B.R., J.E. Rinehart, B.L. Mitchell, C.E. Oakley, C. Carmona, G.L, Gray, and G.S. May. 1987. Cloning, mapping and molecular analysis of the *pyr*G (Orotidine-5'-phosphate decarboxylase) gene of *Aspergillus nidulans.* Gene. 61: 385-399.

Payne, G.A., G.J. Nystrom, D. Bhatnagar, T.E. Cleveland, and C.P. Woloshuk. 1993. Cloning of the *afl*-2 gene involved in aflatoxin biosynthesis from *Aspergillus flavus.* Appl. Environ. Microbiol. 59: 156-162.

Pestka, J.J. 1988. Enhanced surveillance of foodborne mycotoxins by immunoassay. J. Assoc. Off. Anal. Chem. 71: 1075-1081.

Piers, A.C., J.L. Richard, and J.R. Thurston. The influence of mycotoxins on resistance and immunity, in Interactions of mycotoxins in animal production, pp. 56, National Academy of Sciences, Washington, D.C., 1979.

Pitt, J.I. 1993. Corrections to species names in physiological studies on *Aspergillus flavus* and *Aspergillus parasiticus*.

Raper, K.B., and D.I. Fennell. 1965. The Genus Aspergillus, Williams and Wilkins, Baltimore.

Reddy, T.V., L. Viswanathan, and T.A. Venkitasubramanian. High aflatoxin production on a chemically defined medium. Appl. Micro. 22: 393-396.

Rose, M., P. Grisafi., and D. Botstein. 1984. Structure and function of theyeast *URA*3 gene: expression in Escherichia coli. Gene. 29: 113-124.

Schmidt, F.R., P.A. Lemke, and K. Esser. Viral influences on aflatoxin formation by Aspergillus flavus. 1986. Appl. Microbiol. Biotechnol. 24: 248-252.

Schweizer, M., L.M. Roberts, H.-J. Holtke, K. Takabayashi., E. Hollerer, B. Hoffmann, M. Muller, H. Kottig, and E. Schweizer. 1986. The pentafunctional FAS1 of yeast: its nucleotide sequence and order fo the catalytic domains. Mol. Gen. Genet. 203: 479-486.

Sherman, D.H., F. Malpartida, M.J. Bibb, H.M. Kieser, and D.A. Hopwood. 1989. Structure and deduced function of the granaticin-producing polyketide synthase gene cluster of *Streptomyces violaceoruber* Tu22. EMBO J. 8: 2717-2725.

Skory, C.D. 1992. Ph.D. dissertation. Michigan State University, East Lansing.

Skory, C.D., P.-K. Chang, J. Cary, and J.E. Linz. 1992. Isolation and characterization of a gene from *Aspergillus parasiticus* associated with the conversion of versicolorin A to sterigamatocystin in aflatoxin biosynthesis. Appl. Environ. Microbiol. 58: 3527-3537.

Skory, C.D., J.S. Horng, J.J. Pestka, and J.E. Linz. 1990. Transformation of *Aspergillus parasiticus* with a homologous gene *pyr*G involved in pyrimidine biosynthesis. Appl. Environ. Microbiol. 56: 3315-3320.

Skory, C.D., P.-K. Chang, and J. Linz. 1993. Regulated expression of the *nor*-1 and *ver*-1 genes associated with aflatoxin biosynthesis. Appl. Environ. Microbiol. 59: 1642-1646.

Smith, J.E. 1994. *Aspergillus*. Plenum Press, New York.

Stoloff, L. 1989. Aflatoxin is not a probable human carcinogen: the published evidence is sufficient. Regul. Toxicol. Pharmac. 10: 272-283.

Stoner, G.D., F.B. Daniel, K.M. Schenk, H.A.J. Schut, D.W. Sandwisch, and A.F. Gohara. 1982. DNA binding and adduct formation of aflatoxin B1 in cultured human and animal tracheobronchial and bladder tissues. Carcinogenesis. 3: 1345-1348.

Swan, D.G., A.M. Rodriguez, C. Vilches, C. Mendez, and J.A. Salas. 1994. Characterization of a *Streptomyces anitibioticus* gene encoding a type I polyketide

synthase which has an unusual coding sequence. Mol. Gen. Genet. 242: 358-362.

Thom, C., and M.B. Church. 1926. The Aspergilli. Williams and Wilkins, Baltimore.

Trail, F., N. Mahanti, M. Rarick, R. Mehigh, S.-H. Liang, R. Zhou, and J.E. Linz. 1995. Physical and transcriptional map of an aflatoxin gene cluster in *Aspergillus parasiticus* and functional disruption of a gene involved early in the aflatoxin pathway. Appl. Environ. Microbiol. 61: 2665-2673.

Trail, F., P.-K. Chang, J. Cary, and J.E. Linz. 1994. Structural and functional analysis of the *nor*-1 gene involved in the biosynthesis of aflatoxins by *Aspergillus parasiticus.* Appl. Environ. Microbiol. 60: 40784085.

Turner, R.B., D.L. Lindsey, D.D. Davis, and R.D. Bishop. 1975. Isolation and identification of 5,7-dimethoxyisoflavone, an inhibitor of *Aspergillus flavus* from peanuts. Mycophathologia. 57: 39-40.

Vining, L.C. 1992. Secondary metabolism, inventive evolution and biochemical diversity - a review. Gene. 115: 135-140.

Watanabe, I., F. Nara., and N. Serizawa. 1995. Cloning, characterization and expression of the gene encoding cytochrome P-450sca-2 from Streptomyces carbophilus involved in production of pravastatin, a specific HMG-CoA reductase inhibitor. Gene. 163: 81-85.

Wicklow, D.T. 1983. Taxonomic features and ecological significance of sclerotia. Southern cooperative series bulletin. 279: 6-12.

Williams, D.H., M.J. Stone, P.R. Hauck, and S.K. Rahman. 1989. Why are secondary metabolites biosynthesized? J. Natl. Prod. 52: 1189-1208.

Wilson, L.J., C.L. Carmona, and M. Ward. 1988. Sequence of the *Aspergillus niger pyr*G gene. Nucleic Acids Res. 16: 2339.

Woloshuk, C. P., K. R. Foutz, I F. Brewer, D. Bhatnagar, T. E. Cleveland, and G. A. Payne. 1994. Molecular characterization of *afl*-R a regulatory locus for aflatoxin biosynthesis. Appl. Environ. Microbiol. 60: 2408-2414.

Woloshuk, C.P., E.R. Seip, G.A. Payne, and R. Adkins. 1989. Genetic transformation system for the aflatoxin-producing fungus *Aspergillus flavus*. Appl. Environ. Microbiol. 55: 86-90.

Wood, A.H., R.F. Bozarth, J. Adler, and D.W. Mackenzie. 1974. Proteinaceous virus-like particles from an isolate of *Aspergillus flavus*. J. Virol. 13: 532-534.

Wu, T. S., and J.E. Linz. 1993. Recombinational inactivation of the gene encoding

nitrate reductase in *Aspergillus parasiticus.* Appl. Environ. Microbiol. 59: 2998-3002.

Yu, J., J.W. Cary, D. Bhatnagar, T. E. Cleveland, N. P. Keller, and F. S. Chu. 1993. Cloning and characterization of a cDNA from *Aspergillus parasiticus* encoding an O-methyltransferase involved in aflatoxin biosynthesis. Appl. Environ. Microbiol. 59: 3564-3571.